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244. THE VITAMIN A AND CAROTENE CONTENT OF SHORTHORN COLOSTRUM

BY K. M. HENRY, J. HOUSTON AND S. K. KON

From the National Institute for Research in Dairying, University of Reading

IT has been known for some time that cow's colostrum is a richer source of vitamin A than milk (1). More recently Dann (2) studied samples of colostrum from the herd of Shorthorn cows belonging to the Cambridge University Farm and found that in some samples the concentration of vitamin A was as much as 100 times and that of carotene up to 70 times higher than in later milk. As in occasional tests in our laboratory on colostral fats from Shorthorn cows the differences were never so great, it was decided to carry out a more systematic investigation.

EXPERIMENTAL

Samples of colostrum were obtained from Shorthorn cows and heifers calving in the Institute's herd in the course of a year. To measure not only the concentration but also the total yield of vitamin A and carotene the calves were not allowed to suckle, the colostrum was removed quantitatively, an aliquot taken for measurements and the rest fed to the calf. As it was considered inadvisable to strip some of the heavy milking cows completely, these were excluded from the experiment. This accounts in part for the predominance of heifers in these experiments. In all, nine heifers and four cows were studied. The first sample of colostrum was obtained within 5–12 hr. (in one case within 2 hr.) of parturition. Thereafter the yields over 24 hr. were bulked and in this way a further three or four daily samples of colostrum (depending on the time of calving) were obtained. The fourth sample sometimes covered less than 24 hr. Butter was churned from the following first normal milking, from a pooled sample of milk for the ensuing week and from a final 24 hr. sample taken a fortnight later. Fat was extracted from the samples of colostrum by the method described by Gillam *et al.* (3). The vitamin A and carotene in the milk and in butterfat were measured in Moore blue and yellow units as described by Gillam *et al.* (4).

The general feeding and management of the cows was as described by Golding *et al* (5), but none of the cows or heifers had access to cabbage or kale for the last 3 or 4 months before calving. Irrespective of season the cows were kept indoors for a fortnight after calving.

Results

The results are given in Tables 1–3. Table 1 gives particulars of the cows and yields, Table 2 the vitamin A and Table 3 the carotene contents of the samples of colostrum and milk. Cows which had had access to pasture before calving are marked with an obelisk in the tables. In all three tables the cows have been arranged in descending order of the concentration of vitamin A in the first colostrum.

Table 1. *Particulars of cows, yields and fat percentage of samples of colostrum and milk*

No. and name of cow	Calving date	No. of lactation	No. of days dry	1st colostrum		2nd colostrum		3rd colostrum		4th colostrum	
				Yield (kg.)	% fat	Yield (kg.)	% fat	Yield (kg.)	% fat	Yield (kg.)	% fat
1. Lottie 12	8. iii. 37	1	—	3·25	5·1	6·08	4·5	7·83	4·3	8·14	5·4
2. Red Rose 4†	24. ix. 37	1	—	2·47	8·1	4·22	4·2	6·11	1·7	9·32	5·5
3. Peach 13	13. iii. 37	1	—	2·37	6·6	7·52	7·7	7·52	4·1	9·17	4·3
4. Flora 35†	20. v. 38	1	—	2·47	9·5	6·59	4·8	7·83	4·5	8·03	5·8
5. Winsome 14†	26. xi. 37	1	—	1·96	6·1	7·31	5·0	7·73	3·8	8·34	4·8
6. Phoebe	22. i. 38	1	—	1·39	4·9	4·74	4·8	9·27	7·2	9·27	7·3
7. Lily 5	8. iii. 37	1	—	3·71	3·9	8·24	3·7	12·05	5·4	11·23	4·3
8. Lottie 8†	21. vi. 37	3	162	6·58	3·5	13·38	3·2	16·33	3·5	17·01	5·2
9. Pippin 3	3. i. 38	1	—	2·06	3·2	6·90	3·6	8·76	3·1	9·37	2·9
10. Lottie 7†	4. v. 37	3	107	7·14	4·4	10·55‡	3·2	15·88	4·0	16·33	3·6
11. Flora 42	7. iii. 38	1	—	2·47	2·8	8·14	4·3	6·70	4·0	10·71	3·1
12. Campion 3†	22. v. 37	3	244	10·21	2·9	11·79	3·4	12·25	3·3	12·25	3·5
13. Portia 5	21. iii. 38	3	106	3·50	1·8	7·62	3·2	10·20	3·1	11·43	3·2

No. and name of cow	Calving date	No. of lactation	No. of days dry	5th colostrum*		1st milk		2nd milk		3rd milk	
				Yield (kg.)	% fat	Yield (kg.)	% fat	Yield (kg.)	% fat	Yield (kg.)	% fat
1. Lottie 12	8. iii. 37	1	—	9·06	4·8	6·92	4·4	8·68	4·4	9·98	4·2
2. Red Rose 4†	24. ix. 37	1	—	4·64	4·4	9·53	5·6	11·11	4·3	13·49	4·1
3. Peach 13	13. iii. 37	1	—	5·15	3·5	10·77	4·3	11·63	3·5	13·27	3·9
4. Flora 35†	20. v. 38	1	—	5·15	3·8	9·41	5·8	10·53	4·5	12·02	3·6
5. Winsome 14†	26. xi. 37	1	—	—	—	10·21	4·3	11·78	3·8	13·38	3·4
6. Phoebe	22. i. 38	1	—	6·08	3·1	11·23	4·7	12·26	4·1	14·97	3·6
7. Lily 5	8. iii. 37	1	—	—	—	11·11	5·1	12·98	3·7	14·06	3·5
8. Lottie 8†	21. vi. 37	3	162	7·71	4·8	17·24	7·4	17·53	5·3	21·66	3·1
9. Pippin 3	3. i. 38	1	—	9·48	2·4	8·16	2·8	11·61	2·9	12·50	4·3
10. Lottie 7†	4. v. 37	3	107	16·33	3·6	17·01	4·1	18·78	3·4	21·77	2·4
11. Flora 42	7. iii. 38	1	—	—	—	—§	—§	11·42§	2·7§	14·74	3·0
12. Campion 3†	22. v. 37	3	244	6·80	2·8	13·15	2·4	13·59	3·2	15·65	3·1
13. Portia 5	21. iii. 38	3	106	6·39	2·1	14·06	3·9	14·76	3·1	16·78	3·4

* The period of collection of this colostrum varied according to the time of calving; it was 24 hr. for cows 1, 5, 7, 9, 10 and 11; the hours of collection for the remaining cows were: no. 2, 13·5; no. 3, 14; no. 4, 15; no. 6, 14; no. 8, 13; no. 12, 13; no. 13, 15.

† On pasture before calving.

‡ Period of collection 19 hr.

§ The 1st milk and the first 4 days of the 2nd milk were not collected.

Table 2. *The vitamin A content of the samples of colostrum and milk expressed in Moore blue units*

Cow no.	1st colostrum			2nd colostrum		3rd colostrum		4th colostrum	
	Hours after calving	Moore b.u./100 g.	Total output* Moore b.u. × 10^3	Moore b.u./100 g.	Total output* Moore b.u. × 10^3	Moore b.u./100 g.	Total output* Moore b.u. × 10^3	Moore b.u./100 g.	Total output* Moore b.u. × 10^3
1	10·5	8160	264·8	5932	360·5	2611	204·4	1575	128·2
2†	5·0	6479	160·2	2372	100·2	471	28·8	931	86·8
3	12·0	5942	140·8	5544	416·9	1435	107·9	796	72·9
4†	5·0	4749	117·4	1748	115·2	747	58·5	528	42·5
5†	11·0	4444	87·0	2537	185·5	938	72·4	697	58·1
6	9·0	3922	54·6	1920	91·0	1476	136·8	689	63·9
7	10·0	3608	133·8	2628	216·6	1836	221·3	688	77·2
8†	11·5	3588	236·0	1829	244·7	1400	228·6	1423	242·2
9	6·0	3199	65·9	2485	171·5	1042	91·2	638	59·8
10†	8·0	3153	225·3	1614	170·2 (215·0)	1382	219·5	774	126·4
11	10·5	2939	72·7	4037	328·5	1926	128·9	651	69·7
12†	11·0	1532	156·4	1156	136·3	853	104·5	290	35·5
13	2·0	820	28·7	1365	104·0	937	95·5	811	92·7

Cow no.	5th colostrum		1st milk		2nd milk		3rd milk	
	Moore b.u./100 g.	Total output* Moore b.u. × 10^3	Moore b.u./100 g.	Total output* Moore b.u. × 10^3	Moore b.u./100 g.	Total output* Moore b.u. × 10^3	Moore b.u./100 g.	Total output* Moore b.u. × 10^3
1	627	56·8	579	40·0	299	26·0	242	24·1
2†	484	22·4 (62·2)	769	73·3	291	32·4	280	37·8
3	420	21·6 (46·4)	401	43·2	227	26·4	258	34·2
4†	312	16·0 (35·7)	432	40·7	261	27·5	152	18·3
5†	—	—	426	43·5	211	24·8	137	18·3
6	145	8·8 (17·3)	177	19·9	169	20·7	150	22·4
7	—	—	417	46·3	171	22·2	174	19·8
8†	960	74·0 (142·9)	843	145·3	317	55·6	259	56·0
9	344	32·6	396	32·3	176	20·4	171	21·4
10†	310	50·6	328	55·8	201	37·7	123	26·8
11	—	—	—	—	114	13·0	181	26·6
12†	115	7·9 (19·3)	263	34·6	220	29·9	251	39·3
13	344	22·0 (55·8)	468	65·8	196	29·0	175	29·4

* The total outputs are given for 24 hr. periods with the exception of the 1st colostrum, where the period of collection is given in column 2, and of the 5th colostrum, the collection period of which can be seen from Table 1. Calculated figures for 24 hr. yields for the 5th colostrum are given in brackets; this also applies to the 2nd colostrum of cow 10.

† On pasture before calving.

Table 3. *The carotene content of the samples of colostrum and milk expressed in Moore yellow units*

Cow no.	1st colostrum		2nd colostrum		3rd colostrum		4th colostrum	
	Moore y.u./100 g.	Total output* Moore y.u. × 100	Moore y.u./100 g.	Total output* Moore y.u. × 100	Moore y.u./100 g.	Total output* Moore y.u. × 100	Moore y.u./100 g.	Total output* Moore y.u. × 100
1	816	264·8	396	240·4	196	153·0	139	113·4
2†	1331	329·1	535	226·0	106	64·9	236	220·0
3	822	194·6	650	488·7	178	133·5	118	108·4
4†	2026	500·8	550	362·6	326	255·3	271	217·6
5†	1464	286·6	704	514·8	284	219·0	253	211·2
6	621	86·4	235	111·2	216	200·2	113	104·9
7	676	250·6	487	401·0	312	376·2	148	166·1
8†	902	593·5	384	513·8	288	469·9	240	408·3
9	742	152·9	505	348·4	216	188·9	122	114·7
10†	1330	949·8	756	797·3 (1007·0)	456	724·3	248	405·1
11	411	101·5	510	415·0	254	170·3	96	103·3
12†	684	698·2	347	409·8	202	247·6	183	225·2
13	448	156·8	672	512·1	379	386·9	256	292·7

Cow no.	5th colostrum		1st milk		2nd milk		3rd milk	
	Moore y.u./100 g.	Total output* Moore y.u. × 100	Moore y.u./100 g.	Total output* Moore y.u. × 100	Moore y.u./100 g.	Total output* Moore y.u. × 100	Moore y.u./100 g.	Total output* Moore y.u. × 100
1	55	49·9	31	21·3	33	28·6	30	30·0
2†	142	65·7 (135·5)	135	128·6	82	91·4	75	101·2
3	43	22·4 (56·8)	56	60·2	32	37·8	22	28·7
4†	137	70·5 (133·9)	139	131·0	70	73·3	31	37·1
5†	—	—	118	120·1	84	99·0	49	65·0
6	36	21·9 (38·3)	34	38·3	26	32·3	28	42·1
7	—	—	66	72·9	44	57·6	32	36·2
8†	159	122·5 (221·3)	121	208·4	41	72·3	48	104·1
9	67	63·2	73	59·6	51	58·8	32	40·6
10†	124	202·2	69	117·8	32	59·3	60	130·0
11	—	—	—	—	36	41·1	78	114·6
12†	86	58·2 (74·3)	37	48·6	43	58·2	47	72·3
13	131	83·4 (174·1)	126	176·5	75	110·2	61	102·4

* See footnote to Table 2.

† On pasture before calving.

Vitamin A. When the concentration of vitamin A in colostrum is considered there is a marked variation from cow to cow in the highest value recorded, that for cow 1 being ten times as high as that for cow 13. The latter cow showed, however, a greater concentration in her second colostrum, and if this is compared with the value for cow 1 a ratio of 6:1 is obtained. When the total outputs of vitamin A throughout the colostral period are considered

(probably a better method of gauging how variable may be the amount available to the calf), the ratio is reduced to 3:1 (cows 8 and 6). The total outputs can be calculated from data in Table 2. In only two cases (cows 11, 13) was there a higher concentration of vitamin A in the second colostrum than in the first. In following milkings a rapid fall in concentration took place for all cows and by the fifth day the level maintained in the later milk was generally reached. The subsequent rise in the vitamin A level of some of the cows was due to their going out to pasture. In Table 4 the ratios of the highest concentration of carotene or vitamin A in colostrum and colostral fat to their

Table 4. *Highest concentrations of vitamin A and carotene in samples of colostrum and colostral fats and highest daily yields of these factors in colostrum in terms of their lowest respective concentrations and yields in the later milks of the same cows*

	Concentration in colostrum		Concentration in colostral fat		Daily yield	
Cow no.	Vitamin A	Carotene	Vitamin A	Carotene	Vitamin A	Carotene
1	34	27	27	23	25	28
2†	23	18	11	9	24	17
3	23	37	13	21	16*	17*
4†	31	65	13	24	31	65
5†	32	30	18	17	10	10
6	27	24	20	21	7	7
7	21	21	23	22	16	17
8†	14	22	17	32	9	17
9	19	23	25	29	13	15
10†	26	42	14	34	25	48
11	35*	14*	26	11	25*	10*
12†	13	18	14	18	11	31
13	8*	11*	9	14	12	18
Mean	24	27	18	21	17	23

N.B. In each case the lowest concentration in milk is taken as unity.
* 2nd colostrum taken. † On pasture before calving.

lowest concentration in milk or milk fat are given for each cow. The ratio of the highest total daily yield in colostrum to the lowest yield in milk is also given. The highest ratio for vitamin A in colostrum is 35:1, for cow 11. This is one-third of the highest value reported in this country for Shorthorn cows by Dann (2). The average ratio is 24:1. The highest ratio reported by Stewart & Macallum (6) in their comprehensive study of 100 cows, mostly Ayrshires, was 46:1. When the concentrations in the fat are compared the highest and average figures are respectively 27:1 (cow 1) and 18:1. For comparison of total yields the amount secreted in the first colostrum was recalculated on a 24-hourly basis. This, doubtless, weighted the result in favour of the colostrum, as it is not usually to be expected that the rate of secretion of vitamin A and carotene observed in the first few hours after calving would be maintained for the whole twenty-four. Even so, the highest ratio is not more than 31:1 (cow 4) and the average 17:1.

Carotene. Regarding the secretion of carotene (Table 3) it will be seen that in the first colostrum there is a fivefold difference between the highest

and lowest values (cows, 4, 11). The ratio is reduced to 4:1 if the second colostrum of the latter cow is included. The highest and lowest total outputs in the colostral period (which can be calculated from Table 3) are those of cows 10 and 6 and stand in the ratio of 6:1. Cows 11 and 13 show, as with vitamin A, a higher concentration of carotene in the second than in the first colostrum. It will be seen from Table 4 that, as regards the relative concentrations of carotene in colostrum and later milk, the highest ratio is 65:1 for cow 4. This is very similar to the highest ratio recorded by Dann (2). The average for all cows is 27:1. When the concentration in fat is considered the highest ratio is 34:1 (for cow 10) and the average is 21:1. Finally, the calculation of total yields (which favours colostrum, cf. p. 5) gives a maximum ratio of 65:1 (again for cow 4), while the average is 23:1.

Table 5. *Arrangement of cows in descending order of highest concentrations or yields of vitamin A or carotene in colostrum*

Vitamin A/ 100 g. colostrum	Carotene/ 100 g. colostrum	Vitamin A/g. colostral fat	Carotene/g. colostral fat	Total yield in colostral period	
				Vitamin A	Carotene
1	4†	1	*c* 10†	*c* 8†	*c* 10†
2†	5†	11	*c* 8†	1	*c* 8†
3	2†	*c* 8†	*c* 13	*c* 10†	*c* 12†
4†	*c* 10†	9	*c* 12†	3	*c* 13
5†	*c* 8†	7	5†	7	4†
11*	3	3	9	11	5†
6	1	2†	4†	*c* 12†	7
7	9	6	7	2†	3
c 8†	*c* 12†	5†	2†	9	2†
9	7	*c* 10†	1	5†	9
c 10†	*c* 13*	*c* 12†	11	*c* 13	1
c 12†	6	4†	6	4†	11
c 13*	11*	*c* 13	3	6	6

N.B. "*c*" before a number denotes a cow; the rest are heifers.
* 2nd colostrum. † On pasture before calving.

Comparison of cows and heifers. If the figures for the concentration of vitamin A in colostrum or colostral fat are placed in descending order (Table 5), the heifers (with one exception) occupy the highest positions. This agrees with the findings of Dann (2). If, however, the placing is based on total yields, higher positions are taken by cows owing to their larger yields of colostrum.

When the figures for carotene are arranged similarly the cows lead in respect of total yield and of concentration of carotene in the colostral fat but the comparisons are complicated by the effects of pasture feeding.

Access to pasture. It may be noted from Table 5 that access to pasture before calving is not reflected in a higher output of vitamin A whether measured by concentration in colostrum and colostral fat or by total yield. On the other hand, under these circumstances, the secretion of carotene is definitely higher.

Discussion

These results may be compared with the estimations of Dann (2) in England and of Stewart & Macallum (6) in Scotland in which Lovibond Tintometer measurements were also used and the results were expressed in Moore units.

Strictly speaking, a correction for the specific gravity of colostrum should be introduced when comparing our values with those of the other observers. The correction is too small to be of any significance.

For carotene our highest value, just over 2000 y.u. per 100 g. colostrum is more than double Stewart & Macallum's (6) highest value of 800 and is also higher than Dann's (2) maximal figure of 1300. The Scottish experiments were carried out during the stall-feeding period and when our figures for cows and heifers which had access to pasture are excluded (cf. p. 6) none of the remaining values is higher than Stewart & Macallum's maximum.

On the other hand, there is a marked difference in the vitamin A figures. The highest value in Scotland was 1300 b.u. per 100 ml. and only three cows out of 100 showed values above 1000 b.u. Dann's (2) maximal figure is 2800, and nine of his fourteen cows had more than 1000 b.u. per 100 ml. of colostrum. Our maximal value is over 8000 with only one cow below the 1000 mark.

The measurement of the evanescent blue colour given by vitamin A with the antimony trichloride reagent is notoriously more difficult than the colorimetric estimation of carotene, and one might expect differences between laboratories caused by differences in technique. The differences now recorded are, however, too great to be accounted for on this basis. Neither does it seem likely that they could be due solely to differences in breed, feeding and management of the cows. If so, one would expect the discrepancy to extend to the carotene figures. We are inclined to suggest that the marked difference between our figures and those of Stewart & Macallum (6) might possibly be due to the use in the two laboratories of different techniques for the preparation of the samples for the $SbCl_3$ test. We carried out our tests on the non-saponifiable residue of the fatty fraction of colostrum and milk. Stewart & Macallum (6) extracted their colostrum by the alkali digestion method of Davies (7) which does not saponify the fat. It has been shown by Booth *et al.* (8) that, owing to the presence of inhibitors, a direct blue test on butterfat gives much lower values than the test on the non-saponifiable residue. We have found now, for example, that a test on milk carried out by Davies's (7) technique gave 53 b.u. per 100 g., while when the fat extracted by this method was saponified or when butter was churned from the milk and tested by our method the result was 130 b.u. per 100 g. Similar differences were found between saponified and unsaponified samples of colostrum. Davies's (7) method is perfectly satisfactory for the purpose for which it was evolved, i.e. for the estimation of vitamin A in livers. In the method used by Dann (2) the fat was saponified to a large extent (9), and it is not therefore easy to account for the differences between his figures and ours for Shorthorn cows. Through the courtesy of the Director of the Cambridge University Farm we obtained milk from two cows of his herd just before they went out to grass, i.e. when the carotene and vitamin A content would be lowest. Butters were churned from both milks and tested for vitamin A and carotene. The results were very smilar and values of 8 y.u. and 70–100 b.u. per 100 g. of milk were obtained. The milk was also tested in

June when the cows were on pasture and gave figures of 40–50 y.u. and 150 b.u. per 100 g. These latter values agree closely with those reported in the present study. Dann's (2) value for carotene of 20 y.u. per 100 g. of "standard" milk is mid-way between the values found by us for stall-fed and pasture-fed cows. Our lowest vitamin A figure of 70 b.u. per 100 g. for the Cambridge cows exceeds Dann's (2) estimate of 30 b.u. per 100 g. for "standard" milk.

We have not attempted to calculate in international units the vitamin A activity of our samples of colostrum and milk because of the uncertainty attaching to the relative activity of vitamin A and carotene in butter (cf. Booth *et al.* (10)).

Summary

1. The concentration of carotene and of vitamin A in colostrum and colostral fat and the total daily yield of these substances in successive milkings has been studied for four shorthorn cows and nine heifers.

2. The concentration of vitamin A in the first colostrum ranged from 8160 to 820 Moore blue units per 100 g. and that of carotene from 2026 to 411 Moore yellow units per 100 g.

3. The highest and lowest concentrations and yields of vitamin A and carotene respectively in samples of colostrum and later milk were in the ratios: per g. of colostrum (milk) 35:1 and 65:1; per g. of fat 27:1 and 34:1; calculated on daily yield 31:1 and 65:1. This last calculation favours colostrum.

4. Access to pasture before calving appeared to have no effect on the secretion of vitamin A in colostrum but increased the output of carotene.

Our best thanks are due to Mr A. G. Cotton who supervised the collection of the samples of colostrum and to Miss D. V. Dearden for churning the butters.

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Note added 14 September 1939. Since this paper was written we have been informed by Dr Stewart that the method of digestion of colostrum used in his laboratory (6) differed from that of Davies (7) in that 25 ml. of colostrum were heated for 3 hr. with 25 ml. of 24% KOH. It is possible that this technique saponified the fat to some extent. The problem is being investigated by Dr Stewart.

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245. THE PARTITION OF SERUM CALCIUM ABOUT THE TIME OF PARTURITION IN THE DAIRY COW

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There does not appear to be any record in the literature of the complete fractionation of serum calcium at the time of parturition. It was felt that a complete study of the partition of serum calcium during the period of transient reduction in serum calcium associated with parturition should be undertaken to remedy this deficiency. Also it was thought that studies of the changes in the different serum calcium fractions during times of fluctuating total calcium levels might contribute to our knowledge of the interrelations of the different fractions.

While the fluctuations in the various calcium fractions of serum are of considerable theoretical interest, there is a wide practical application. The dairy cow is subject to a variety of pathological conditions in which hypocalcaemia is an outstanding characteristic. In the following communication we show that the physiologically available calcium in the blood plasma of the dairy cow is lower than in other species on record, and it has been felt that a connexion exists between this and the frequency with which hypocalcaemic conditions arise.

As has been previously emphasized, the dairy cow may simultaneously be absorbing calcium from the intestine, depositing calcium in or mobilizing it from the skeleton, secreting calcium in the mammary gland, transporting calcium across the placenta and filtering calcium off in the kidneys. Consequently a knowledge of how calcium is transported in the vascular system is essential for adequate interpretation of calcium metabolism. Further, calcium has definite functions to perform in normal tissue processes.

The methods of complete fractionation of serum calcium have been introduced only recently, and this, together with the long and somewhat tedious analytical procedure, has resulted in there being very little data with which to compare our results. There is also, as is to be expected, a lack of cognate information in collateral fields. For these reasons some of our data must be regarded, for the time being, as merely on record.

Analytical methods

When drawing a sample of jugular blood, we use an "oiled" needle and collect the blood in a scrupulously clean tube after discarding the first 5 ml. or so of blood. Collection in this manner results in the almost complete absence of any tissue fluid, and it is normally several hours before coagulation is complete. Bodansky (1) has called attention to a "paradoxical" increase in serum

phosphatase activity of samples allowed to stand at room temperature. The increase can be considerable and, for this reason, we have modified and standardized our method of serum preparation so that each sample can be placed in the incubator for phosphatase estimation exactly 1 hr. from the time of sampling. It occurred to us that coagulation might be accelerated by the addition of "glass-wool" to the sample, since such a material would provide centres for the precipitation of fibrin. The method proved quite satisfactory, and we now add a small ball of "glass-wool" to each sample, the ball being thrust just below the surface of the blood sample. Other workers have reported and we have observed that serum samples frequently deposit traces of fibrin during the calcium estimation and are, thereby, a source of error. Methods have been devised to avoid this difficulty. We have found that with bovine blood "glass-wool" ensures the rapid and complete precipitation of fibrin. In cases where "glass-wool" was not used we have found incomplete precipitation after as much as 5 hr. standing.

Serum calcium partition

The methods used have been reported elsewhere (Duckworth & Godden (2)).

Serum inorganic phosphorus and phosphatase

The method of Bodansky (1) was used.

Results

Table 1. *Serum inorganic phosphorus, serum phosphatase and serum calcium partition in cows at parturition. Values expressed in mg. per* 100 *ml. serum*

Days from calving	Time	Inorganic P	Phos-phatase	T. Ca	Ca^{++}	U. Ca C.	N.U.Ca C.	P.B.Ca
				Cow 1				
5	0940	6·06	1·7	11·3	1·2	4·5	2·3	3·3
3	1010	5·38	1·7	11·3	1·5	4·8	1·7	3·3
2	0930	5·32	1·8	11·2	1·5	4·6	1·8	3·3
0	0245*	3·68	1·9	11·0	1·3	3·9	1·6	4·2
0	0940	6·46	1·9	11·1	1·0	4·7	2·2	3·2
2	0930	3·58	1·9	10·3	1·6	3·8	1·4	3·5
4	0940	4·20	1·6	11·1	1·8	4·3	1·8	3·2
7	0940	6·48	1·7	11·7	1·6	5·1	2·2	2·8
				Cow 2				
0	1145	3·68	3·0	11·6	1·6	3·8	2·2	4·0
0	1630*	2·42	3·1	10·7	2·0	3·3	1·1	4·3
1	1010	3·04	2·3	10·7	1·9	3·6	1·4	3·8
3	1015	3·94	2·7	10·9	1·7	4·1	1·3	3·8
7	0945	5·72	3·4	11·6	1·4	5·0	2·0	3·2
				Cow 3				
3	0950	5·22	3·2	11·4	1·4	4·9	1·6	3·5
1	0930	5·46	3·8	11·3	1·2	5·0	1·5	3·6
0	1200	4·48	3·4	11·5	1·5	5·0	1·6	3·4
0	2100*	3·22	3·4	10·3	1·8	3·6	1·6	3·3
1	0945	5·54	2·9	10·7	1·3	4·5	1·6	3·3
4	0945	5·32	2·8	11·2	1·5	5·2	1·6	2·9

Table 1 (*continued*)

Days from calving	Time	Inorganic P	Phos-phatase	T. Ca	Ca++	U. Ca C.	N. U. Ca C.	P. B. Ca
				Cow 5				
8	0945	5·58	4·0	11·1	1·5	5·0	1·5	3·1
6	0935	5·28	3·8	11·5	1·6	4·9	1·4	3·6
0	1030	3·12	3·6	10·5	2·1	3·6	1·3	3·2
0	1430*	2·72	3·6	10·4	2·2	3·6	1·4	3·2
1	0950	4·28	3·6	10·5	1·6	4·2	1·7	3·0
				Cow 6				
4	1015	—	—	10·1	1·1	5·2	1·7	2·1
2	0930	—	—	10·6	1·2	4·9	2·1	2·4
1	1730	—	—	10·9	1·8	4·8	1·5	2·8
0	0400*	—	—	10·6	2·4	4·0	1·5	2·7
1	1100	—	—	10·2	1·6	4·5	1·6	2·5
				Cow 7				
2	0950	6·46	2·3	11·0	1·3	4·4	2·6	2·7
0	1005	4·76	2·4	10·5	1·3	4·6	1·8	2·8
0	1405	3·44	2·6	9·8	1·4	4·0	1·4	3·0
0	1505*	3·94	2·7	10·1	1·6	3·7	1·6	3·2
0	1840	4·20	2·0	10·1	1·3	4·2	1·5	3·1
1	0940	5·16	2·1	10·0	1·3	4·2	1·9	2·6
5	0930	4·86	2·4	9·0	1·0	3·9	1·5	2·6
				Cow 9				
1	1110	3·96	3·5	10·0	1·6	3·8	1·3	3·3
0	0700	3·08	3·9	10·2	2·0	3·7	1·0	3·5
0	0930	2·44	3·8	9·8	1·7	3·1	1·3	3·7
0	1100*	2·70	3·7	9·8	1·5	3·6	0·6	4·1
1	0940	5·02	5·3	10·1	1·2	4·2	1·6	3·1
				Cow 11				
0	0945	—	—	10·7	1·1	4·5	1·7	3·4
0	1730*	—	—	9·8	1·3	3·7	0·5	4·3
1	1010	—	—	9·6	1·5	4·0	1·1	3·0
4	0930	—	—	10·6	1·3	4·6	2·0	2·7
				Cow 12				
2	1030	—	—	11·7	1·8	5·0	1·6	3·3
1	2230	—	—	11·3	1·9	4·9	1·2	3·3
0	0430*	—	—	10·3	—	—	—	—
2	1030	—	—	9·6	1·2	4·5	1·6	2·3
5	0900	—	—	9·7	1·4	4·1	2·0	2·2
				Cow 13				
1	0945	—	—	11·0	1·2	4·5	2·3	3·0
0	0700	—	—	9·4	1·6	3·4	1·1	3·3
0	1010*	—	—	9·2	1·6	3·4	0·9	3·3
1	1015	—	—	10·8	1·9	3·7	3·0	2·2
3	0930	—	—	9·6	1·5	4·4	1·3	2·4

* Denotes time of calving.

In order to obtain sufficient data on the changes in serum calcium partition prior to calving a number of samples were analysed which have been excluded from the records in this communication owing to the restrictions of space. The excluded data were remarkably regular, and such fluctuations as were observed did not appear to have any significance. Copies of the complete data will be made available to interested workers.

All the calvings were normal. Cow 6 had rather a poor appetite after calving and cow 12 retained the afterbirth.

Serum inorganic phosphorus. The reduction in the levels of this constituent in the serum of cows near to calving which was observed in this experiment has been reported by a number of workers.

Serum phosphatase. The changes in serum phosphatase were not marked. Such changes as were observed were usually slight and were probably of no significance.

Total calcium (T. Ca). This herd was the subject of a long-term study of normal changes in blood composition (including calcium partition), and hence a comparison can be made between the data immediately prior to and subsequent to calving and those obtained some weeks before and after calving. All the values found for total serum calcium were higher a few days before calving than those found either a few weeks before or after calving. At the actual time of calving, however, there was generally a reduction of about 10% in the total calcium. One cow (no. 1) showed practically no reduction and another (no. 13) showed a reduction of about 20%.

Calcium ion (Ca^{++}). The values obtained for this fraction were generally, but not always, maximal at or near the time of calving.

Ultrafiltrable calcium complex (U. Ca C.). The values for the U. Ca C. all showed a reduction at the time of calving.

Non-ultrafiltrable calcium complex (N. U. Ca C.). The level of this constituent was always reduced at the time of calving. The reductions were irregular. In cow 11 a value of 0·5 mg. was found, while in cows 1 and 3 the samples taken close to parturition were only reduced to 1·6 mg.

Protein-bound calcium (P. B. Ca). This fraction was generally, but not invariably, increased near the time of calving.

Physiologically available calcium. In the following communication we record that the percentage of ultrafiltrable calcium is lower in the dairy cow than in other species studied. Near the time of parturition there is a tendency for the ultrafiltrable calcium to account for a somewhat higher percentage of total calcium than in previous or subsequent periods.

Discussion

Almost all the cows showed a moderate reduction in serum calcium at the time of calving. Although the reduction was not marked, being generally of the order of 10%, it is important, since the changes which occurred in the quantities of the different calcium fractions bore no simple relation to this reduction. The changes in the various fractions were more marked than would have been expected from the small diminution in total calcium and were consistent from one animal to another.

For some time before calving the serum calcium was at higher levels than those existing some weeks earlier. This increase was reflected chiefly in increases in Ca^{++} and P. B. Ca. Close to the time of calving Ca^{++} generally increased and with it were usually found increases in P. B. Ca. It has been shown elsewhere (Duckworth & Godden(2)) by *in vitro* and *in vivo* studies

that a close relation exists between Ca^{++} and P. B. Ca, and that P. B. Ca can be regarded as a reserve of calcium ions. Under conditions where the level of free calcium ions is depressed there is an immediate mobilization of P. B. Ca and, conversely, when the amount of free calcium ions increases a portion becomes combined with serum proteins. Thus the changes occurring close to calving which result in a liberation of calcium ions cause increases in both Ca^{++} and P. B. Ca. Since cases occur in which there was only a slight increase in either Ca^{++} or P. B. Ca, while the other fraction showed a large increase, the equilibrium between the two cannot be entirely dependent on the level of free calcium ions and serum proteins alone. That other factors are involved seems clear. However, irrespective of the distribution of calcium between Ca^{++} and P. B. Ca, the "actual plus potential" calcium ion (i.e. Ca^{++} + P. B. Ca) increased at the time of calving.

The source of the increases in Ca^{++} and P. B. Ca is apparent from a study of the serum inorganic phosphorus data at the time of calving. The reduction in this constituent was accompanied by a reduction in the U. Ca C. Benjamin (3) has shown that this complex contains phosphate ion and, consequently, the marked reduction in phosphate in the presence of a slight reduction in total serum calcium resulted in the level of U. Ca C. falling. This reduction thereby freed calcium ions which were, as explained above, distributed between Ca^{++} and P. B. Ca. It therefore follows that, in the case of three out of the four forms of calcium occurring in serum, the distribution is mainly influenced by the level, not only of total serum calcium, but also of serum inorganic phosphorus.

The N. U. Ca C. behaved in a manner similar to that observed by Rây (4) in his studies of the effect of different dietaries on the partition of serum calcium. In his experiments, in the present communication and in other data subsequently to be published, there is generally a similarity in behaviour of the N. U. Ca C. and serum inorganic phosphorus. The relation between the two is not consistent, but a reduction in inorganic phosphorus, if of moderately large magnitude, is generally, but not always, accompanied by a reduction in N. U. Ca C. This relation cannot be direct because only under exceptional conditions does the inorganic phosphate of serum become non-ultrafiltrable, and, in such cases, this is the result of formation of colloidal calcium phosphate. Since there is no opportunity of this occurring in our studies, the explanation of the reduction in the N. U. Ca C. must be sought elsewhere.

The absence of noteworthy changes indicates that the blood phosphatase levels are no indication of mammary gland activity. Although this enzyme plays an important, but at present obscure, role in bone formation, there is no good reason to expect it to be involved in the mobilization of osseous reserves of calcium and phosphorus which occurs with the inception of lactation. Consequently, since such changes as were found were slight and appeared to have no relation to the sudden awakening of mammary activity, they are probably best attributed to normal day-to-day variation.

SUMMARY

Data have been given of the variations in the calcium ion, ultrafiltrable calcium complex, non-ultrafiltrable calcium complex and protein-bound calcium at normal parturition in the dairy cow, and an interpretation of the results has been presented. The variations observed have been shown to be consistent for all the animals studied.

Serum phosphatase did not show any consistent changes at the time of parturition.

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246. A LONG-TERM STUDY OF THE PARTITION OF SERUM CALCIUM IN AYRSHIRE COWS

By W. GODDEN and J. DUCKWORTH

From the Rowett Institute, Aberdeen

(With 1 Figure)

In the course of an investigation into the changes occurring in the partition of serum calcium associated with various pathological conditions and with parturition in the dairy cow it was considered desirable, for purposes of comparison, to endeavour to establish normal values for the different serum calcium fractions as no records of such values or ranges existed in the literature.

The work was carried out on a herd of fourteen young Ayrshire cows, each having completed one pregnancy prior to the commencement of the experiment. Blood samples were drawn at varying intervals over a period of 3½ years. The data for samples drawn near to a calving data are not embodied in this communication, since changes associated with parturition are the subject of another paper.

Analytical procedure

The serum calcium was fractionated according to Benjamin & Hess (1), these workers having, by using a technique involving ultrafiltration and adsorption, succeeded in dividing this constituent into four fractions:

(1) Ionic calcium (Ca^{++}).

(2) An ultrafiltrable adsorbable calcium complex (U. Ca C.).

(3) A non-ultrafiltrable adsorbable calcium complex (N. U. Ca C.).

(4) Calcium in combination with, presumably, serum proteins and generally referred to as "protein-bound" calcium (P. B. Ca).

Certain workers have not agreed with Benjamin (2) and with Benjamin & Hess (1), and Benjamin (3) has published a reply to these criticisms. Elsewhere we have published (Duckworth & Godden (4)) physicochemical studies of the partition of serum calcium which led us to support the views of Benjamin & Hess. Recently Rây (5) has presented studies of changes occurring under various experimental conditions in the serum calcium partition in sheep, goats and cows which support our previous observations.

All calcium estimations were made according to the method of Clark & Collip (6). Benjamin & Hess (1), working with small amounts of serum, were compelled to use a colorimetric method for the estimation of calcium. As large amounts of blood were available we were able to use the more accurate titrimetric method.

Barium sulphate, as adsorbent, was prepared according to the method of Benjamin & Hess (1), and the fractionation carried out as recommended by these authors. All samples of adsorbent were checked against each other to ensure similarity of adsorptive capacity. The membranes used for ultrafiltration were prepared and used as described by Duckworth (7).

Results

The results are given in Tables 1–6.

Total serum calcium (Table 1). The values obtained during the first 14 months of the experiment were remarkably regular both at any one bleeding date and usually in individual cows from sampling to sampling. The values obtained at the last two samplings were much higher than those previously

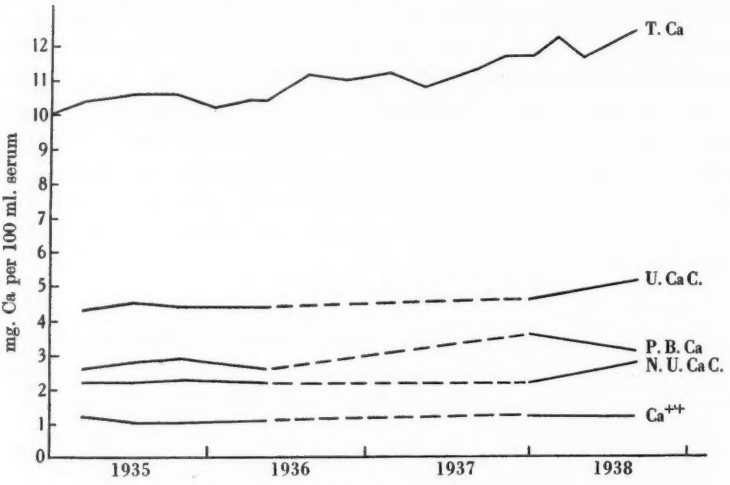

Fig. 1. A long-term study of serum calcium in Ayrshire cows.

found. This increase, with maturity, was contrary to what we had expected, but the regularity with which the increase was found makes the result quite definite. Also it should be observed that the increase persisted over a period of 8 months as shown by the present data. It should be mentioned at this point that the cattle were regularly sampled (at short intervals between the samplings for partition) for the determination, among other constituents, of total serum calcium. The increase in calcium noted at the fifth partition sampling arose gradually between the fourth and fifth samplings. The values for total calcium over the whole experiment are given in Fig. 1.

Calcium ion (Table 2). The average calcium ion values were remarkably constant throughout the whole period. A study of the individual data indicates a greater variation amongst the animals at any one bleeding as they became more mature.

Table 1. *Total serum calcium*

Mg. Ca per 100 ml. serum

Cow no.	25. iii. 35	11. vii. 35	23. x. 35	14. v. 36	17. i. 38	19. ix. 38
1	10·9	10·8	10·5	10·2	10·4	12·7
2	11·4	11·0	11·0	10·2	11·2	12·7
3	10·2	10·7	10·8	10·6	12·5	13·9
4	10·0	10·7	11·5	10·4	11·8	12·0
5	10·6	10·5	10·8	10·3	11·4	12·6
6	10·8	10·6	10·5	10·9	11·8	—
7	10·2	10·2	10·5	10·1	11·3	12·1
8	10·0	10·3	10·1	9·5	10·6	12·4
9	10·4	10·7	10·4	11·3	12·4	12·4
10	10·4	10·5	10·5	10·8	11·8	12·2
11	10·2	10·6	10·4	10·8	12·5	11·5
12	9·8	10·3	10·6	9·7	11·9	12·0
13	10·5	10·5	10·3	10·7	12·6	12·2
14	10·2	10·5	10·3	—	—	—
Average	10·4	10·6	10·6	10·4	11·7	12·4

Table 2. *Calcium ion*

Mg. Ca per 100 ml. serum

Cow no.	25. iii. 35	11. vii. 35	23. x. 35	14. v. 36	17. i. 38	19. ix. 38
1	1·3	1·1	0·9	1·0	1·3	0·8
2	1·3	1·0	1·2	1·0	1·3	1·2
3	1·3	1·1	1·1	1·1	1·3	1·8
4	1·3	1·0	1·2	1·1	1·1	—
5	1·2	1·0	1·1	1·0	1·3	1·0
6	1·3	1·0	1·0	1·1	1·5	—
7	1·2	1·0	1·0	1·0	1·1	0·8
8	1·2	1·0	0·8	1·0	1·0	0·8
9	1·2	1·1	0·9	1·3	1·2	1·4
10	1·1	1·2	0·8	1·1	1·2	1·1
11	1·2	1·2	0·9	1·2	1·3	1·1
12	1·2	1·0	0·8	1·4	1·3	1·0
13	1·2	1·0	1·1	1·4	1·3	2·0
14	1·1	1·0	0·8	—	—	—
Average	1·2	1·0	1·0	1·1	1·2	1·2

Table 3. *Ultrafiltrable calcium complex*

Mg. Ca per 100 ml. serum

Cow no.	25. iii. 35	11. vii. 35	23. x. 35	14. v. 36	17. i. 38	19. ix. 38
1	4·5	4·4	4·4	4·4	4·6	6·3
2	4·6	4·4	5·0	4·2	4·6	5·4
3	4·2	4·1	4·7	4·8	5·0	5·5
4	4·2	4·6	4·7	4·6	4·6	—
5	4·6	4·7	4·8	4·3	4·0	5·5
6	4·6	4·5	4·7	4·5	4·2	—
7	4·4	4·5	4·9	4·2	4·2	5·4
8	4·5	4·5	4·7	4·0	3·9	5·6
9	4·4	4·4	4·0	4·6	4·7	5·2
10	4·0	4·7	4·3	4·4	4·5	5·2
11	4·1	4·3	4·2	4·7	5·0	4·4
12	4·2	4·5	4·0	4·1	4·8	5·3
13	4·2	4·8	4·1	4·5	5·1	4·9
14	3·9	4·6	3·8	—	—	—
Average	4·3	4·5	4·4	4·4	4·6	5·3

Table 4. *Non-ultrafiltrable calcium complex*

Mg. Ca per 100 ml. serum

Cow no.	25. iii. 35	11. vii. 35	23. x. 35	14. v. 36	17. i. 38	19. ix. 38
1	2·4	2·5	2·8	2·6	1·2	3·2
2	2·5	2·5	2·0	2·3	1·6	2·3
3	2·1	2·4	2·1	2·1	1·6	2·5
4	2·3	2·3	1·5	2·1	2·6	—
5	2·6	1·8	2·1	2·2	2·6	2·7
6	2·1	2·2	2·1	2·6	1·9	—
7	2·4	2·0	2·1	2·2	2·8	3·4
8	2·1	2·1	2·0	1·7	2·7	2·7
9	2·2	2·4	2·6	2·4	2·6	3·0
10	2·5	2·2	2·5	2·4	2·5	2·4
11	2·3	2·3	2·5	2·3	2·2	3·1
12	1·8	2·1	2·8	2·0	2·4	3·6
13	1·7	1·3	2·2	2·2	2·7	1·5
14	2·5	2·1	3·0	—	—	—
Average	2·2	2·2	2·3	2·2	2·2	2·8

Table 5. *Protein-bound calcium*

Mg. per 100 ml. serum

Cow no.	25. iii. 35	11. vii. 35	23. x. 35	14. v. 36	17. i. 38	19. ix. 38
1	2·7	2·8	2·4	2·2	3·3	2·4
2	3·0	3·1	2·8	2·7	3·7	3·8
3	2·6	3·1	2·9	2·6	4·6	4·1
4	2·2	2·8	4·1	2·6	3·5	—
5	2·2	3·0	2·8	2·8	3·5	3·4
6	2·8	2·9	2·7	2·7	4·2	—
7	2·2	2·7	2·5	2·7	3·2	2·5
8	2·2	2·7	2·6	2·8	3·0	3·3
9	2·6	2·8	2·9	3·0	3·9	2·8
10	2·8	2·4	2·9	2·9	3·6	3·5
11	2·6	2·8	2·8	2·6	4·0	2·9
12	2·6	2·7	2·9	2·2	3·4	2·1
13	3·4	3·4	2·9	2·6	3·5	3·8
14	2·7	2·8	2·7	—	—	—
Average	2·6	2·8	2·9	2·6	3·6	3·1

Table 6. *Physiologically available calcium*

Values expressed as percentages of total serum calcium

Cow no.	25. iii. 35	11. vii. 35	23. x. 35	14. v. 36	17. i. 38	19. ix. 38
1	53	51	50	53	57	56
2	52	49	56	51	53	52
3	54	49	54	56	50	53
4	55	52	51	55	49	—
5	55	54	55	51	47	52
6	55	52	54	51	49	—
7	55	54	56	51	47	51
8	57	53	54	53	46	52
9	54	51	47	52	48	53
10	49	56	49	51	48	52
11	52	52	49	55	50	48
12	55	53	45	57	51	52
13	51	55	50	55	51	57
14	49	53	45	—	—	—
Average	53	53	51	53	50	53

Ultrafiltrable calcium complex (Table 3). The values found for this fraction were remarkably constant in individuals and at any one bleeding in early life. As with the ionic calcium, irregularity within groups and inconsistencies in the same individual occur with maturity.

Non-ultrafiltrable calcium complex (Table 4). Although a fair degree of regularity existed at each sampling in immature cows, some individuals showed wide changes from sampling to sampling. The irregularities became more marked with increasing age.

Protein-bound calcium (Table 5). The values found for this fraction were irregular at any one bleeding and for the same individual at different dates. The irregularities increased with age.

Physiologically available calcium (Table 6). It is probably correct to view the ultrafiltrable calcium as that which is immediately physiologically available. The non-ultrafiltrable calcium, or at least the P. B. Ca, acts as a circulatory reserve of calcium which is immediately available to remedy any transitory depression in ultrafiltrable calcium. In Table 6 is given the physiologically available (i.e. ultrafiltrable) calcium expressed as a percentage of total serum calcium.

Apart from the results at the fifth sampling, where the values tended to be lower, the average values at each sampling are uniform throughout the period. There appears to be rather more variation both for individuals over the period and within the group at any one bleeding.

DISCUSSION

The four series of samples drawn in 1935–6 showed quite constant average levels for total serum calcium, and this regularity was associated with a similar constancy in the average values for the four calcium fractions. The increase found at the fifth sampling was associated with an increased level of P. B. Ca. This increase in P. B. Ca without a corresponding increase in Ca^{++} is unexpected, for, since our previous studies indicated that a depression in Ca^{++} caused a mobilization of P. B. Ca, we should have expected a somewhat parallel movement of the two fractions. It would seem that other factors are involved in this equilibrium. The further increase at period 6 was accompanied by a reduction in P. B. Ca, the difference in total calcium being accounted for by increases in both U. Ca C. and N. U. Ca C.

The following ranges have been established for normal dairy cows both young and mature:

Calcium ion. An average of about 10–12 % of the total serum calcium is in the ionic form. This value is somewhat lower than that of Brinkman & Van Dam (8) and Neuhausen & Marshall (9), who found a calcium-ion level of about 20 % of the total serum calcium. On the other hand, Sendroy & Hastings (10) have expressed the opinion that the level is probably closer to 10 %. While our value agrees with Sendroy & Hastings we would emphasize that our study was

restricted to the bovine over a period of barely one-quarter of the normal life span.

Ultrafiltrable calcium complex. Generally 40–45 % of the serum calcium is combined in this form.

Non-ultrafiltrable calcium complex. About 20 % of the serum calcium is combined in this form. The behaviour of this fraction is more erratic than the U. Ca C., although individuals appear to maintain fairly constant levels for long periods of time.

Protein-bound calcium. About 25 % of the serum calcium is combined in this form.

The fluctuations observed in the different serum calcium fractions become more marked with increasing age. It appears that factors are involved which are not yet clear. Since no differences were noted clinically between animals, and since the animals used in this experiment were in normal health (as judged by general appearance, reproduction and lactation performance), it appears that the variations we have found are normal.

The finding that only 50–53 % of the serum calcium is physiologically available (i.e. ultrafiltrable) is surprising in view of the higher values generally found for other species. This point may be of fundamental importance, since the dairy cow, more commonly than all other animals, is prone to conditions characterized by depressed serum calcium and, therefore, depressed ultrafiltrable calcium.

The dairy cow has the most continually active calcium transport mechanism of any animal. Calcium may be transported simultaneously for lactation, foetal ossification and development and either to or from the skeletal reserves. Under such conditions, it is not remarkable that fluctuations should occur in total serum calcium and, therefore, in the partition of serum calcium. It would probably not be suggested that the distribution of the serum calcium between the different fractions was anything other than a physicochemical relationship, but the present data and those obtained in studies subsequently to be published, together with the recent communication of Rây (5), make it clear that the factors involved are still very imperfectly understood.

Summary

The following average fractionation of serum calcium in the dairy cow has been reported:

Ca^{++}	10–12 %
U. Ca C.	40–45 %
N. U. Ca C.	About 20 %
P. B. Ca	About 25 %
Ultrafiltrable Ca	50–53 %

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247. THE PROBLEM OF VARIATIONS IN THE GROWTH-PROMOTING VALUE OF MILK FOR RATS

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(With 4 Figures)

It has been reported by Elvehjem *et al.* (1) that rats receiving an exclusive diet of summer milk supplemented with minerals grow better than rats receiving milk from stall-fed cows. They also found that, while pasteurization had little deleterious effect on the growth-promoting properties of summer milk, those of "winter" milk were markedly reduced. Experiments carried out in this laboratory (2,3) failed to confirm the above findings. The technique in our work was different, however, from that used by Elvehjem *et al.*, who fed their rats *ad libitum*, in that the intakes of the milks were equalized. We also subsequently learned (4,5) that Elvehjem *et al.* specially prepared their rats for the tests.

Experiments involving exclusive milk feeding *ad lib.* in which this special technique was used as a means of detecting nutritional differences between milks, have since been carried out by us (6,7) as yet without success. None of these later experiments involved, however, a comparison of "winter" and summer milk, and our earlier experiments have therefore been repeated and extended. Two series of experiments were carried out. In the first rats received exclusively milk with added minerals, in the second cane sugar was also added.

Kohler *et al.* (8) have shown that guinea-pigs are unable to subsist on a diet of mineralized "winter" milk alone but can grow satisfactorily when fresh grass juice is added to the milk. If, as is believed by these workers (4,7), the "grass-juice factor" is responsible for the difference in growth-promoting properties between "winter" and summer milk, one would expect guinea-pigs to grow better or to remain alive longer on pasture milk than on "winter" milk. For this reason summer and "winter" milk was also fed to guinea-pigs.

Experimental

(1) *Source and preparation of the milks*

Three pairs of Shorthorn cows were selected for the experiment, the two animals in each pair being matched as well as possible for age, milk yield, date of calving, and pre-experimental treatment. One animal from each pair was then allocated by lot to the indoor treatment and its partner to the normal treatment. Details concerning the animals are shown in Table 1.

Table 1. *Particulars of cows*

Name of cow	Treatment	No. of calves produced	Date of last calving	Approx. initial milk yield 26. iii. 39 lb. per day	Approx. final milk yield 9. vii. 39 lb. per day
Phoebe	Indoors	2	29. xii. 38	25·5	19·9
Doris 3	Normal	4	26. xii. 38	37·7	31·1
Flora 31	Indoors	4	26. i. 39	33·2	35·4
Rosalie 9	Normal	2	10. ii. 39	32·5	21·7
Dorrit 5	Indoors	1	17. ii. 39	28·0	25·7
Flora 48	Normal	1	21. ii. 39	25·6	23·4

(*a*) *Feeding and housing.*

The object of the rationing was to give the "normal" group a ration typical of those used under practical farming conditions in England (i.e. winter rations followed by a sudden change to grazing in spring), and by way of contrast to keep another group of cows indoors on the winter ration without grass throughout the winter, spring and early summer.

During the autumn of 1938 the ration of the animals consisted of a gradually decreasing amount of pasture grass with a corresponding increase in other foods—hay, straw, kale and concentrates. Only a negligible amount of grass was available after mid-November, and after 18 December, with the onset of sharp frosts and snow, no more kale or other green food was available. From 19 December onwards the ration consisted of hay, straw, mangels and concentrated foods, the quantities being varied slightly according to the dates of calving of the cows. Within 14 days of calving the ration of each cow was standardized to the following: medium quality meadow hay 12–14 lb., mangels (mixed white fleshed globes and red intermediates) 40 lb., and concentrated foods (consisting of a mixture of crushed oats, flaked wheat, flaked maize, maize germ meal and decorticated ground nut cake) 4 lb. per gallon of milk produced.

The "indoor" group of cows was kept indoors and fed this ration continuously till 10 July 1939. The "normal" group received the same ration till spring grass was available; the details of grazing were as follows:

The inherent fertility of the pasture land used in this experiment is normally inferior to that of high-grade English pasture, due to lack of drought resistance, but during the spring of 1939 no spells of drought occurred and growth of grass was abundant and well above the average for the district.

The "normal" group of cows were allowed on a small almost bare pasture by day from 3 to 19 April; from 20 April to 2 May they were given access to good pasture for 6½ hr. daily. During this period the winter ration was reduced by omitting mangels and half the hay. There is little doubt that the fresh pasture grass eaten supplied more than half the food requirements of the cows. From 3 May onwards the "normal" cows were on good pasture night and day, the only supplement being a small ration of concentrates. Frequent changes of pasture were made and the supply of grass was ample to satisfy the cows.

(*b*) *Details of composition of milk.*

The rat feeding experiments were started on 8 May and continued until 10 July. Experiments on guinea-pigs lasted from 10 May to 12 June.

The method of blending the morning and evening milks from the two groups of cows to a common fat percentage and details of subsequent pasteurization have been fully described in (3). Estimations of fat and of total solids were carried out daily on the blended samples of milk. The mean values are given in Table 2.

Table 2. *Mean figures for fat and total solids in the blended samples of summer and "winter" milks*

	% fat	% total solids
Summer milk	3·26	12·10
"Winter" milk	3·26	11·96

Samples of butter were churned from the experimental milks three times during the course of the experiment. Their vitamin A and carotene contents were estimated by means of Lovibond Tintometer tests as described by Booth *et al.* (9) and Gillam *et al.* (10). The results of these tests are given in Table 3.

Table 3. *Vitamin A and carotene contents of the experimental milks expressed as Moore blue and yellow units*

	Summer milk			"Winter" milk	
Period	Moore blue units/g. fat	Moore yellow units/g. fat	Period	Moore blue units/g. fat	Moore yellow units/g. fat
6–7. v. 39	57·1	21·3	3–4. v. 39	25·0	2·9
8–9. vi. 39	57·1	16·9	8–9. vi. 39	25·0	2·9
3–4. vii. 39	58·6	14·7	3–4. vii. 39	12·8	3·0

Chemical estimations of vitamin B_1 and of riboflavin were also done. The first was measured as described by Houston & Kon (11), the second according to Emmerie (12). The results will be found in Tables 4 and 5.

Table 4. *Vitamin B_1 content of the experimental milks*

	International units per 100 ml.	
Date	Summer milk	"Winter" milk
18. iv. 39	11·1	11·8
28. iv. 39	11·3	11·1
12. v. 39	10·1	10·1
26. v. 39	6·3	10·1
9. vi. 39	8·5	9·6
22. vi. 39	9·0	9·8
10. vii. 39	9·0	11·0

Table 5. *Riboflavin content of the experimental milks*

Date	μg./100 ml. Summer milk	"Winter" milk
18. iv. 39	151·2	116·5
28. iv. 39	131·3	95·3
12. 5. 39	125·0	90·1
26. v. 39	177·9	121·8
9. vi. 39	163·1	110·1
22. vi. 39	146·1	113·3
10. vii. 39	163·1	112·2

(2) *The feeding of the milks*

Rats were prepared for the experiment according to the technique of Kohler *et al.* (5). The mothers received the usual stock diet of this laboratory (13), but milk from the indoor group of cows was fed to them from the time the pups were born, and they were not given liver during the period of lactation.

The guinea-pigs used in the experiments were bred in the Institute. While with the dam they were gradually weaned on to the stock meal mixture, which they were given exclusively when 3 weeks old, together with unlimited drinking water, unlimited hay and a small daily allowance of fresh cabbage. The composition of the stock diet is given in Table 6.

Table 6. *Composition of stock diet for guinea-pigs*

Bran	27
Oats	27
Lucerne meal	13
Linseed cake	13
Flaked maize	13
Fish meal	7

(*a*) *Exclusive feeding to rats of milk supplemented with minerals.*

Twelve litters of four male rats were placed at weaning in individual cages. The animals in each litter were assigned by lot to either (i) raw summer milk, (ii) pasteurized summer milk, (iii) raw "winter" milk, or (iv) pasteurized "winter" milk. The milks were fed *ad lib.* according to the technique described by Bartlett *et al.* (6). In the course of the first week of the experiment two litters were reduced to three animals owing to the death of one animal on raw summer and of another on pasteurized "winter" milk. Both animals had been scouring badly and were oedematous. The litter-mates of these two animals were subsequently killed and the groups replaced by two further groups of animals aged 23 and 24 days which had been kept from weaning in reserve on a diet of raw "winter" milk. The rats received the experimental milks for 8 weeks, when they were killed as usual with coal-gas and the body lengths measured according to Donaldson (14). A post-mortem examination was also carried out on each animal. Rough bi-weekly records of scouring were kept. All animals scoured up to the tenth day of the experiment, subsequently only two animals on pasteurized "winter" milk scoured slightly.

The results of this experiment are shown in Fig. 1, which gives the growth curves, and in Table 7 in which the gains in weight and body lengths of the rats together with their milk intakes are set out. One rat on pasteurized "winter" milk, no. 8661, died of pneumonia after it had been on experiment for

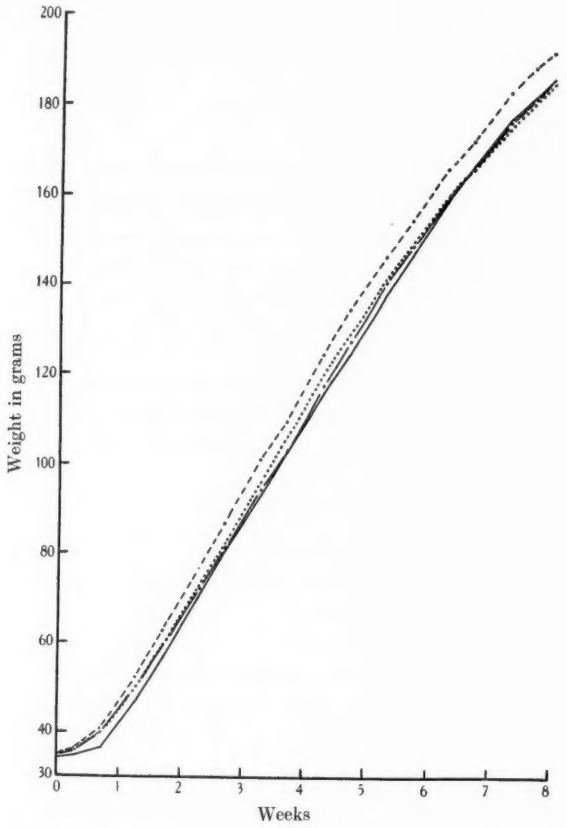

Fig. 1. Average growth curves (11 ♂♂ each) of rats receiving *ad lib.* the experimental milks supplemented with minerals.

•••••• Raw summer milk. – – • – – Pasteurized summer milk.
•—•—• Raw "winter" milk. ——— Pasteurized "winter" milk.

10 days. Two sets of mean figures are given, one for twelve rats and the other for eleven where the litter-mates of the dead animal are omitted.

It will be seen that the mean gains in weight of the four groups of experimental animals are very similar. The animals receiving the two types of pasteurized milk made slightly better gains than those receiving the raw milks. The figures show further that there was very little difference in appetite

Table 7. *Individual and mean figures for gains in weight and milk intakes of the rats receiving, as an exclusive diet, the experimental milks supplemented with minerals*

Litter no.	Rat no.	Weight of rat (g.) Initial	Final	Gain	Gain per day g.	Milk intake ml.	Solid intake g.*	g. gain per g. solid intake	Body length mm.
				Raw summer milk					
I	8563	34	218	184	3·3	4704·5	569·2	0·32	213
II	8572	40	291	151	2·7	4253·5	514·7	0·29	202
III	8594	36	184	148	2·6	4212·0	509·7	0·29	200
IV	8613	38	177	139	2·5	4178·5	505·6	0·27	203
V	8631	37	193	156	2·8	4051·0	490·2	0·32	204
VI	8650	34	209	175	3·1	4523·0	547·3	0·32	206
VII	8656	33	160	127	2·3	3700·0	447·7	0·28	191
VIII	8666	32	184	152	2·7	4125·5	499·2	0·30	194
IX	8680	34	178	144	2·6	3880·5	469·5	0·31	199
X	8693	32	148	116	2·1	3513·0	425·1	0·27	183
XI*a*	8740	30	187	157	2·8	4115·5	498·0	0·32	197
XI*b*	8744	34	184	150	2·7	4429·0	535·9	0·28	205
Mean		34·5	184·4	149·9	2·68	4140·5	501·0	0·298	199·8
Mean omitting litter VIII		34·7	184·5	149·7	2·68	4141·9	501·2	0·297	200·3
				Pasteurized summer milk					
I	8564	35	213	178	3·2	4896·0	592·4	0·30	207
II	8568	40	190	150	2·7	4071·0	492·6	0·30	199
III	8590	36	194	158	2·8	3962·0	479·4	0·33	203
IV	8616	36	204	168	3·0	4779·0	578·3	0·29	208
V	8632	34	168	134	2·4	3632·0	439·5	0·30	194
VI	8651	34	208	174	3·1	4720·5	571·2	0·30	209
VII	8653	33	148	115	2·1	3460·5	418·7	0·27	187
VIII	8660	36	174	138	2·5	3834·0	463·9	0·30	190
IX	8679	36	190	154	2·8	3938·5	476·6	0·32	200
X	8694	30	195	165	2·9	4275·5	517·3	0·32	208
XI*a*	8745	32	207	175	3·1	4397·0	532·0	0·33	200
XI*b*	8743	37	187	150	2·7	4080·0	493·7	0·30	201
Mean		34·9	189·8	154·9	2·78	4170·5	504·6	0·305	200·5
Mean omitting litter VIII		34·8	191·3	156·5	2·80	4201·1	508·3	0·305	201·5
				Raw "winter" milk					
I	8565	36	229	193	3·4	5168·5	618·2	0·31	205
II	8570	41	177	136	2·4	4034·0	482·5	0·28	194
III	8595	37	160	123	2·2	3595·5	430·0	0·29	191
IV	8617	36	171	135	2·4	3841·5	459·4	0·29	196
V	8633	34	145	111	2·0	3170·0	379·1	0·29	186
VI	8648	36	221	185	3·3	5008·0	599·0	0·31	206
VII	8657	35	173	138	2·5	4109·5	491·5	0·28	196
VIII	8665	36	179	143	2·6	4275·0	511·3	0·28	192
IX	8681	35	186	151	2·7	4114·5	492·1	0·31	201
X	8695	31	163	132	2·4	3714·5	444·3	0·30	195
XI*a*	8741	31	195	164	2·9	4305·5	514·9	0·32	196
XI*b*	8742	36	221	185	3·3	4881·0	583·8	0·32	207
Mean		35·3	185·0	149·7	2·68	4184·8	500·5	0·298	197·1
Mean omitting litter VIII		35·3	185·6	150·3	2·68	4176·6	499·5	0·300	197·6
				Pasteurized "winter" milk					
I	8561	36	176	140	2·5	4215·0	504·1	0·28	197
II	8569	36	181	145	2·6	3935·5	470·7	0·31	196
III	8592	36	160	124	2·2	3369·0	402·9	0·31	191
IV	8620	38	192	154	2·8	4405·5	526·9	0·29	202
V	8635	36	175	139	2·5	3786·5	452·9	0·31	197
VI	8649	36	211	175	3·1	4666·5	558·1	0·31	209
VII	8654	32	150	118	2·1	3658·0	437·5	0·27	189
VIII	8661	33	—	—	—	—	—	—	—
IX	8682	34	212	178	3·2	4696·5	561·7	0·32	208
X	8692	30	192	162	2·9	4435·5	530·5	0·31	200
XI*a*	8739	30	203	173	3·1	4661·0	557·5	0·31	196
XI*b*	8746	32	190	158	2·8	4387·5	524·7	0·30	205
Mean omitting litter VIII		34·2	185·7	151·5	2·71	4201·5	502·5	0·302	199·1

* Calculated from the data in Table II.

between the groups as judged by the voluntary consumption of milk. This is even more marked when the total solid intake is considered. The efficiency of utilization of the milk measured by gain in weight per g. total solids consumed was also the same for all the groups and, finally, the mean body lengths were practically identical. The obvious lack of difference between groups makes a statistical analysis superfluous. The mean daily gain in weight of the rats in this experiment was 2·7 g. This is very much the same as figures of 2·5 and 2·8–2·9 g. found by us previously (6, 7) in similar experiments.

There was rather more "wheeziness" among the rats belonging to the "winter" groups, but post-mortem examination showed nothing abnormal in the lungs, with the exception of two rats in the raw summer milk group and of one animal in each of the other three groups, whose lungs were patchy.

(*b*) *Feeding to rats of milk containing dissolved sugar and supplemented with minerals.*

Twelve litters of four male rats, prepared as for the exclusive milk-feeding experiment, were placed in individual cages at weaning. As usual they were distributed by lot among the four milks. 20 g. of castor sugar were added to each 100 ml. milk; this produced 114 ml. of the milk and sugar solution, which was fed *ad lib.* From the third week onwards the animals were given water to drink.

The results of this experiment are given in Tables 8 and 9 and in Fig. 2. Table 8 gives the individual and mean figures for the gains in weight and data on the milk intakes of the experimental animals. Table 9 deals with the statistical treatment of some of the differences. Two sets of mean figures will be found in Table 8, one set is for twelve rats and the other for eleven. This omits the litter-mates of rat 8639 which died when it had been on the experiment for 9 days, probably through milk getting into its lungs. As on previous occasions (6, 15) the addition of sugar to the milks increased the growth rate of the rats in all groups. It will be seen that the animals receiving the summer pasteurized milk made the best gains (3·4 g. daily), while the "winter" pasteurized group made the poorest gains (2·9 g. daily). Reference to Table 9*a* shows that this difference and that between summer raw and "winter" pasteurized milk are statistically significant, but that the other differences between the milks are not significant. When allowance is made for differences in total solid intake (Table 9*c*), it is found in addition that more economical gains were made on summer pasteurized than on "winter" raw milk. Fig. 3 shows that growth quickened after the first 3 weeks of the experiment. For the last 4 weeks the daily gains in weight were 3·8, 3·6, 3·5 and 3·2 g. respectively for summer pasteurized, "winter" raw, summer raw and "winter" pasteurized milk.

As in the exclusive milk feeding experiments there were a few more cases of "wheeziness" on the "winter" milks. Abnormal lungs were found in only

one rat on pasteurized summer and in two on pasteurized "winter" milk. The rats receiving the addition of sugar looked very healthy and, if anything, had better and silkier coats than the animals fed exclusively with mineralized milk.

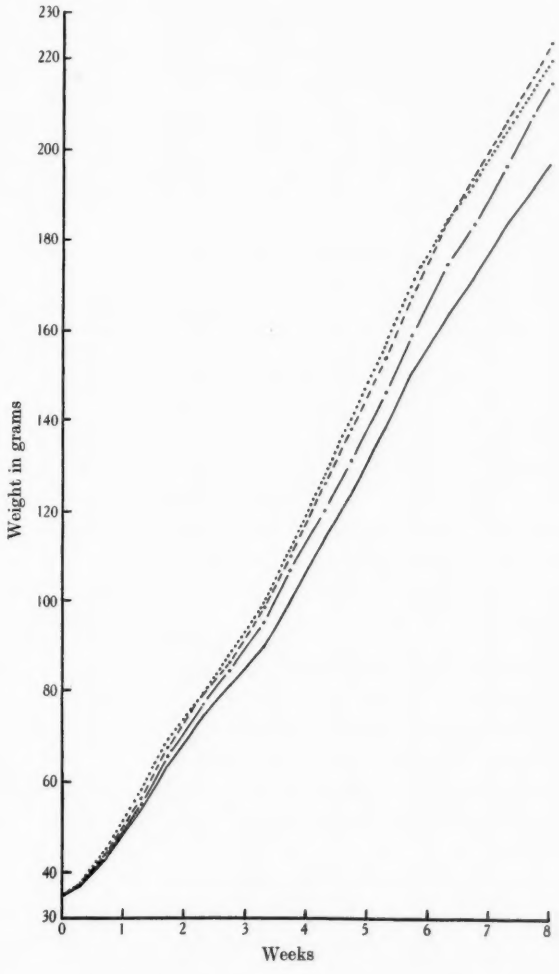

Fig. 2. Average growth curves (11 ♂♂ each) of rats receiving *ad lib.* the experimental milks with added sugar and supplemented with minerals.

•••••• Raw summer milk. --•-- Pasteurized summer milk.
•—•—• Raw "winter" milk. ——— Pasteurized "winter" milk.

Table 8. *Individual and mean figures for gains in weight and food intakes of the rats receiving, as an exclusive diet, the experimental milks + sugar supplemented with minerals*

Litter no.	Rat no.	Weight of rat (g.) Initial	Final	Gain	Gain per day g.	Milk + sugar solution intake ml.	Total solid intake g.*	g. gain per g. solid intake	Body length mm.
				Raw summer milk					
I	8559	38	222	184	3·3	2591·0	729·6	0·25	202
II	8578	36	228	192	3·4	2616·0	736·6	0·26	204
III	8604	41	257	216	3·9	2782·5	783·5	0·28	216
IV	8612	39	212	173	3·1	2490·0	701·1	0·25	207
V	8618	34	250	216	3·9	2794·5	786·9	0·27	214
VI	8623	36	152	116	2·1	1833·5	516·3	0·22	186
VII	8639	32	—	—	—	—	—	—	—
VIII	8658	36	223	187	3·3	2706·5	762·1	0·25	208
IX	8667	30	191	161	2·9	2207·0	621·5	0·26	195
X	8675	33	244	211	3·8	2928·5	824·6	0·26	210
XI	8690	30	212	182	3·3	2440·5	687·2	0·26	201
XII	8707	33	216	183	3·3	2623·5	738·8	0·25	207
Mean omitting litter VII		35·1	218·8	183·7	3·30	2546·7	717·1	0·255	204·5
				Pasteurized summer milk					
I	8558	38	215	177	3·2	2560·5	721·0	0·25	200
II	8579	36	247	211	3·8	2895·0	815·2	0·26	211
III	8601	35	189	154	2·8	2165·0	609·6	0·25	200
IV	8608	36	189	153	2·7	2215·0	623·7	0·25	196
V	8615	34	255	221	3·9	2767·0	779·1	0·28	212
VI	8624	35	234	199	3·6	2733·5	769·7	0·26	213
VII	8643	36	230	194	3·5	2618·5	737·3	0·26	206
VIII	8659	36	200	164	2·9	2400·0	675·8	0·24	201
IX	8663	31	240	209	3·7	2717·0	765·1	0·27	205
X	8669	33	236	203	3·6	2637·5	742·6	0·27	208
XI	8691	34	235	201	3·6	2695·5	759·0	0·26	209
XII	8708	34	211	177	3·2	2491·0	701·4	0·25	203
Mean		34·8	223·4	188·6	3·38	2574·6	725·0	0·258	205·3
Mean omitting litter VII		34·7	222·8	188·1	3·36	2570·6	723·8	0·258	205·3
				Raw "winter" milk					
I	8562	37	195	158	2·8	2449·5	686·7	0·23	205
II	8580	32	187	155	2·8	2122·5	595·1	0·26	192
III	8603	38	191	153	2·7	2244·0	629·1	0·24	198
IV	8611	38	240	202	3·6	2773·0	777·4	0·26	209
V	8614	35	227	192	3·4	2639·0	739·9	0·26	209
VI	8621	33	193	160	2·9	2300·5	645·0	0·25	198
VII	8638	32	177	145	2·6	2224·0	623·5	0·23	194
VIII	8655	36	224	188	3·4	2816·5	789·6	0·24	210
IX	8664	31	214	183	3·3	2603·0	729·8	0·25	203
X	8674	33	220	187	3·3	2546·0	713·8	0·26	201
XI	8689	37	242	205	3·7	2805·0	786·4	0·26	211
XII	8709	33	219	186	3·3	2740·0	768·2	0·24	202
Mean		34·6	210·8	176·2	3·15	2521·9	707·0	0·248	202·7
Mean omitting litter VII		34·8	213·8	179·0	3·20	2549·0	714·6	0·250	203·5
				Pasteurized "winter" milk					
I	8560	38	239	201	3·6	2850·5	799·1	0·25	207
II	8577	32	172	140	2·5	2195·0	615·4	0·23	195
III	8602	37	206	169	3·0	2276·5	638·2	0·26	206
IV	8607	38	201	163	2·9	2493·5	699·1	0·23	208
V	8619	36	210	174	3·1	2386·0	668·9	0·26	205
VI	8627	34	140	106	1·9	1782·0	499·6	0·21	185
VII	8640	34	180	146	2·6	2124·0	595·4	0·25	194
VIII	8652	36	180	144	2·6	2257·0	632·8	0·23	199
IX	8662	31	165	134	2·4	1926·5	540·1	0·25	188
X	8668	33	188	155	2·8	2245·5	629·5	0·25	200
XI	8688	34	211	177	3·2	2477·0	694·5	0·25	205
XII	8705	34	248	214	3·8	2970·5	832·7	0·26	219
Mean		34·7	195·0	160·3	2·87	2332·0	653·7	0·244	200·9
Mean omitting litter VII		34·8	196·4	161·6	2·89	2350·9	659·1	0·244	201·5

* The intake of sugar is calculated on the basis that 100 ml. milk + 20 g. sugar gave 114 ml. of milk + sugar solution; the solids in the milks are calculated from data in Table II.

Table 9. *Statistical treatment of the differences observed in the gains in weight, the total solid intakes and the gains/g. solid intake of the animals receiving the experimental milks and sugar supplemented with minerals*

Difference tested	Numerical difference	Standard error of mean	P*	
(*a*) Gains in weight (g.)				
Summer raw – summer pasteurized	– 4·4	±11·5	1 : 2	Not significant
"Winter" raw – "winter" pasteurized	+15·9	± 9·1	1 : 9	Not significant
Summer raw – "winter" raw	+ 4·7	± 9·9	1 : 2	Not significant
Summer pasteurized – "winter" pasteurized	+28·3	±12·2	1 : 25	Significant
Summer raw – "winter" pasteurized	+22·1	± 8·8	1 : 33	Significant
Summer pasteurized – "winter" raw	+12·4	± 8·9	1 : 4	Not significant
(*b*) Total solid intakes (g.)				
Summer raw – summer pasteurized	– 6·7	±36·6	1 : 1	Not significant
"Winter" raw – "winter" pasteurized	+53·3	±26·5	1 : 14	Not significant
Summer raw – "winter" raw	+ 2·5	±31·0	1 : 1	Not significant
Summer pasteurized – "winter" pasteurized	+71·3	±37·3	1 : 12	Not significant
Summer raw – "winter" pasteurized	+58·0	±28·3	1 : 15	Not significant
Summer pasteurized – "winter" raw	+18·0	±30·2	1 : 2	Not significant
(*c*) g. gain in weight/g. solid intake				
Summer raw – summer pasteurized	–0·003	±0·005	1 : 2	Not significant
"Winter" raw – "winter" pasteurized	+0·004	±0·006	1 : 2	Not significant
Summer raw – "winter" raw	+0·005	±0·005	1 : 3	Not significant
Summer pasteurized – "winter" pasteurized	+0·014	±0·005	1 : 62	Significant
Summer raw – "winter" pasteurized	+0·011	±0·003	1 : 156	Significant
Summer pasteurized – "winter" raw	+0·010	±0·003	1 : 88	Significant

* P = probability that a mean difference at least as great as the observed mean difference would have arisen by random sampling from a homogeneous population.

(*c*) *Experiments with guinea-pigs.*

Twenty-four male guinea-pigs weighing 275–320 g. were used for these experiments. The animals were paired off as regards weight, six pairs were given raw "winter" or summer milk, and the other six pairs received the raw milks and sugar solutions. The animals were given daily 1 ml. of the salt mixture as used for the rats from the third week onwards (1) and an aqueous solution containing 2 mg. of ascorbic acid. These solutions were fed first thing in the morning mixed with 5 ml of the appropriate milk. When this was consumed more milk was offered *ad lib.* Sterilized wood shavings were given as bedding. It was proposed to carry out the experiments for 8 weeks, but none of the animals survived for this period. Their weight curves will be found in Figs. 3 and 4, and the findings are summarized in Table 10. The milk intake was poor, being usually less than 100 ml./day with the plain milk (one animal took on one occasion as much as 140 ml. of the "winter" milk), and about 50–60 ml. daily for the milk and sugar solution. The guinea-pigs receiving only mineralized milk survived on the average 7½ days on the summer milk and 14 days on the "winter" milk. The longest survival in this group was 4 weeks. The animals lived longer when sugar was added to the milk, on the average 19 days on the summer milk and 15½ on the "winter". All the animals suffered

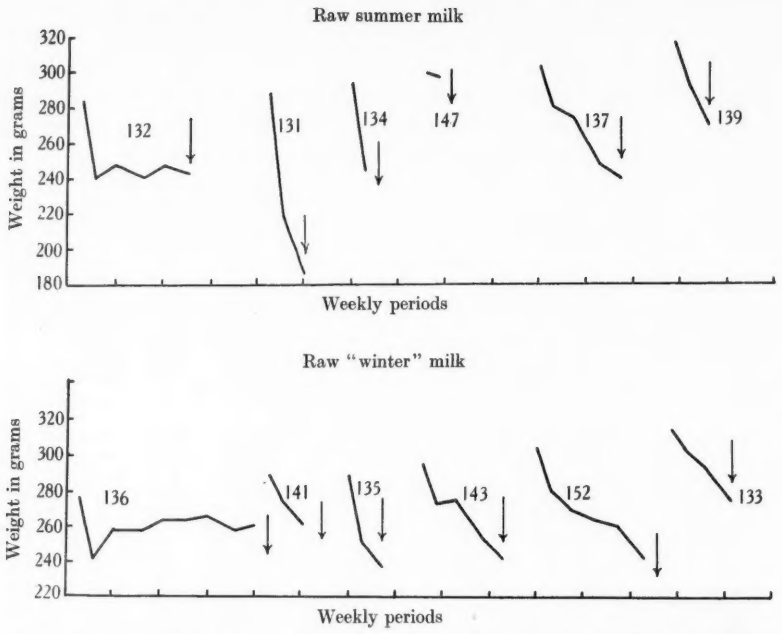

Fig. 3. Individual growth curves of guinea-pigs receiving an exclusive diet of raw summer or "winter" milk supplemented with minerals. Deaths indicated by arrows.

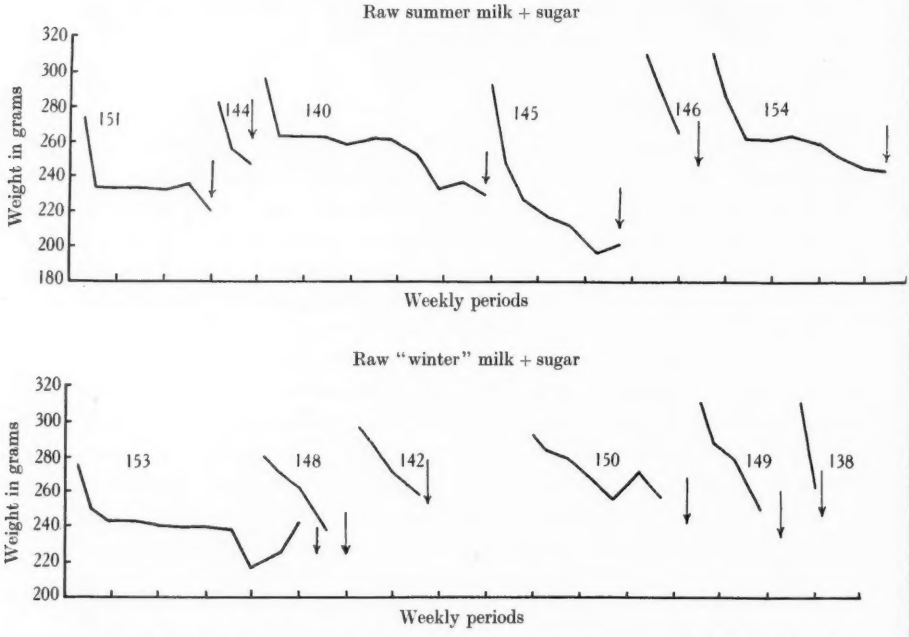

Fig. 4. Individual growth curves of guinea-pigs receiving raw summer or "winter" milk with added sugar and supplemented with minerals. Deaths indicated by arrows.

Table 10. *Individual and mean figures for the weights, food intakes and days of survival of guinea-pigs receiving an exclusive diet of mineralized milk with or without sugar*

Mineralized milk

Guinea-pig no.	Weight of guinea-pig (g.) Initial	Final	Loss	Days of survival	Milk intake ml.
		Summer			
132	283	243	40	16	944
131	288	185	103	5	93
134	294	244	50	3	40
147	300	298	2	3	128
137	304	240	64	12	901
139	317	269	48	5	277
Mean	297·7	246·5	51·2	7·3	397·2
		"Winter"			
136	277	261	16	29	2760
141	290	263	27	8	607
135	289	249	40	8	459
143	296	243	53	12	874
152	306	244	62	18	1314
133	317	277	40	9	557
Mean	295·8	256·2	39·6	14·0	1095·2

Mineralized milk + sugar

Guinea-pig no.	Weight of guinea-pig (g.) Initial	Final	Loss	Days of survival	Intake of milk + sugar solution (ml.)
		Summer			
151	274	220	54	19	900
144	282	247	35	7	230
140	296	229	67	34	1547
145	292	200	92	19	725
146	309	263	46	8	362
154	310	242	68	26	1284
Mean	293·8	233·5	60·3	18·8	841·3
		"Winter"			
153	275	241	34	36	1890
148	280	238	42	11	466
142	296	258	38	10	408
150	292	257	35	22	1086
149	310	250	60	11	410
138	311	264	47	3	42
Mean	294·0	251·3	42·7	15·5	717·0

an immediate loss in weight. This then became stationary or decreased more gradually. No appreciable gains were ever made.

These results are similar to those obtained by Kohler *et al.* (8), who found that guinea-pigs would not live long on an exclusive diet of mineralized milk. They show, moreover, that as far as the survival of guinea-pigs receiving such a diet is concerned, the best summer milk is, under our conditions, no better than milk from cows which had been stall fed for a considerable time.

Discussion

In this experiment we have followed as carefully as possible the technique which allowed the Wisconsin workers to demonstrate differences between milk produced on pasture and milk from stall-fed cows. It is well recognized that pasture milk is much richer in certain factors than winter milk, but the findings of the American authors were attributed by them not to such causes but to a new specific factor present in rapidly growing fresh grass and believed by them to pass into summer milk.

An essential difference exists, however, between experiments on milk carried out in this country and in the United States in that the feeding and management of the cows are not the same. A comparison of the Wisconsin fodders (1) with those used by us shows that the former contained corn silage, whereas mangels were used in England. Again the differences in the conditions of growth and curing of hay in the two countries may be of importance; also the ingredients of the concentrate mixture were different. Another point of difference was the nature of the pasture which, under English conditions in general and in the experiment reported here, was permanent grassland, whereas in the United States temporary grassland is more common. These differences may or may not have influenced the results, and in the absence of proof it can be stated that our findings need not necessarily be regarded as refuting or confirming the American work. They show, however, whether a given technique is of value in this country for the assessment of the nutritive properties of milk, and whether the presence in our pasture milk of a new essential factor not found in the milk of stall-fed cows, can be demonstrated by this technique.

From this angle the results now obtained are negative. We found no difference in the appetite and in the growth of rats which received exclusively the best pasture milk available in this district and of rats given milk from cows which had been stall-fed for 6 months. Whether the milks were fed raw or pasteurized made no difference.

Even when the milk intake was nearly halved and the consumption of essential factors likewise reduced by the addition of sugar to the diet, rats receiving the "winter" milk grew just as well and had just as good appetite as those given the summer milk (Table 9*a*, *b*).

Pasteurization was also without effect: if anything, the pasture milk was improved by the treatment; the "winter" milk deteriorated, but neither effect was statistically significant. It was only when summer milk was compared with the "winter" pasteurized that significant differences in gain in weight and in economy of gain (but not in appetite) were found. While this result is of definite interest and deserves further investigation it is not in itself germane to the problems raised in the present enquiry.

Lastly, tests on guinea-pigs also gave negative results. Kohler *et al.* (8) have found these animals especially susceptible to the lack of the grass-juice factor, which is identical, according to them, with the factor present in summer milk.

We have confirmed the findings of these authors that guinea-pigs cannot live on an exclusive diet of mineralized milk, but in our experience they survived rather longer on "winter" than on pasture milk. The addition of sugar to milk prolonged the time of survival, but there was no difference between the milks. As the addition of the grass-juice factor allows guinea-pigs to thrive for long periods of time on mineralized milk, one would expect summer milk, if it were richer in this factor, to produce better growth and survival than "winter" milk.

Summary

1. Three Shorthorn cows were grazed on the best available permanent pasture when the growth of grass was vigorous, and three other cows of similar lactational history continued to be stall-fed. At the time of the experiment they had been on winter rations for 6 months.

2. By suitable blending of morning and evening milkings the fat content of the milk from both groups of cows was adjusted daily to a common level. A part of each milk was then holder-pasteurized.

3. Four groups of specially prepared litter-mate male rats were fed *ad lib.*, for a period of 8 weeks, on raw and pasteurized milks, both being supplemented with minerals. There were no differences in appetite or gain in weight.

4. In similar experiments sugar was added to the milks to double their caloric value. Again the growth and appetite of the animals were the same on the raw summer and "winter" milks. Pasteurization did not alter significantly the value of the milks, but the summer milk, whether raw or pasteurized, was superior to the pasteurized "winter" milk.

5. Guinea-pigs receiving the raw mineralized milks alone or with sugar died within a comparatively short time. There was no difference in growth performance or time of survival between the summer and "winter" milk groups.

6. These experiments give no indication of the presence of a specific new appetite or growth factor in pasture milk.

Acknowledgements

We are greatly indebted to Mr J. Houston and Mr S. Y. Thompson for the vitamin B_1 and riboflavin tests and to Mr A. Wagstaff for the fat analyses.

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Note added 29 January 1940

Johnson *et al.* (*J. Nutrit.* 1939, **18**, 527) have just reported satisfactory growth of two guinea pigs receiving milk from cows fed phosphoric acid alfalfa silage and of one receiving summer pasture milk.

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248. HAEMOLYTIC ORGANISM ISOLATED FROM PASTEURIZED CREAM

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During experiments on the distribution of bacteria in milk and milk products under conditions prevailing in a commercial creamery, it was noticed that the plate count of a pasteurized cream, which had been agitated in a large butter churn (in daily use in the creamery and apparently in a good sanitary condition) appeared to be abnormally high (Table 1). On plating, pin-point colonies, practically invisible to the naked eye, were found to be present in large numbers after 4 days at 21° C. As these colonies were absent from the plates inoculated with similar dilutions of the same cream taken prior to pouring into the churn, it was considered desirable to determine, as far as possible, the nature of these contaminants.

Table 1

	Cream A (2)	Cream B (3)
Plate count (1) (av. counts per 1 ml. for a number of samples)	23,000	48,000,000

(1) Medium: Beef extract-tryptone-glucose-agar; incubation temperature 21° C. for 4 days.
(2) Cream A: Pasteurized at 87·8° C. flash, cooled and held in a closed vat, fitted with a mechanical stirrer for 16 hr. at 0–1·1° C. prior to churning.
(3) Cream B: Obtained by subjecting cream A to 24–30 revolutions in the churn.

The circumstances of their occurrence and the small size of the colonies on a medium (viz. beef extract-tryptone-glucose-agar) which is in general use in our laboratory and is found to be quite satisfactory for the growth of most organisms, suggested that the organism giving rise to the high counts for cream B might be a type of *Str. thermophilus*. This organism, as described by Orla-Jensen (1) and Sherman & Stark (2), is known to be fastidious in its nutritive requirements and has a high minimum growth temperature. Preliminary tests (i.e. growth on blood-agar medium and action on sugars), however, revealed that the tiny colonies were all of one type, and that the organism could possibly be classified as a variety of either *Str. bovis* or *Str. thermophilus*. In an endeavour to establish the identity of this organism the work described here was undertaken.

Technique and methods of study

The stock cultures of the organism were carried in (a beef extract-tryptone-gelatine medium[1]) 0·3% beef extract, 0·5% tryptone, 12% gelatine, the reaction being *p*H 7·2. In order to ensure vigorous growth prior to inoculating

[1] This medium has also been found very suitable for carrying stock cultures of *Str. agalactiae*

into fermentable media and other test substances, transfers were made from the stock cultures to a broth medium consisting of 0·6 % beef extract, 1 % tryptone, and 0·5 % NaCl (in 10 c.c. portions) at *p*H 7·2, to which was added sterile cow-blood serum to a concentration of 1 %. After incubation at 37° C. for 24 hr., good growth was obtained in these cultures; transfers and inocula (0·01 c.c. in the fermentation and hydrolytic tests) from them invariably gave satisfactory results.

In studying fermentation reactions a basal medium, *p*H 7·2, in 10 c.c. portions and consisting of 0·3 % beef extract, 1 % tryptone, and 0·5 % NaCl was used. The test substances were added to the sterile basal medium in a concentration of 0·5 %. Preliminary tests indicated that incubation at 37 or 45° C. would give equally good results. However, a temperature of 37° C. was finally adopted and reactions were recorded after 3 and 7 days. The final *p*H was determined colorimetrically.

The haemolytic powers of the organism were tested on human and ox blood agar of a composition:

Tryptone meat extract (Lemco) agar, *p*H 7·2 ...	100 ml.
Defibrinated blood	5 ml.

Suitable dilutions for the blood plates were prepared from the 24–30 hour old serum broth mother culture as described above, and the nature and type of haemolysis noted after 40–48 hr. incubation at 37° C. Results were recorded following the methods of Brown (3).

In determining the ability of the organism to hydrolyse sodium hippurate, the medium recommended by Ayers & Rupp (4) was used except that peptone was replaced by tryptone to ensure better growth. In testing for the presence of the products of hydrolysis of sodium hippurate, 0·5 ml. of a 50 % sulphuric acid solution was added to 2 ml. of the culture which had been incubated at 37° C. for 3 days. (A control tube was treated and tested similarly.) A white crystalline precipitate, benzoic acid, indicates a positive result; no change, with the exception of a very faint clouding, occurs in the control and negative cultures.[1]

Action on aesculin was tested on Diernhofer's (5) medium. Cultures were tested after 3 and 7 days' incubation at 37° C. by the addition of 0·5 ml. of a 1 % ferric citrate solution to 10 ml. of culture broth.

The methylene-blue milk (1 : 20,000) was freshly prepared. Litmus milk was used in studying the growth of the organism at different temperatures, viz. 15, 21, 45 and 50° C. These cultures were observed every day and all changes noted.

Tests for the ability to liquefy gelatine were made on a medium of composition: gelatine 15 %, bacto-tryptone 0·5 %, and casein (Hammerstein's)

[1] This method of testing for the products of hydrolysis of sodium hippurate, while perhaps not as sensitive a test as the usual method of adding 7 % ferric chloride, has given satisfactory results and has been found to be very convenient as a test in routine work especially with mastitis streptococci.

0·5 %. The incubation temperature was 37° C., and results were recorded after 3 and 7 days, the tubes being allowed to stand at room temperature for a period of 2 hr. after removal from the incubator.

The effect of NaCl upon the growth of the organism was studied in a lactose-tryptone-broth medium to which the salt was added prior to inoculation, to give concentrations of 2, 2·5 and 3 % respectively. The resistance of the organism to high temperatures was tested in plain sterile milk and in lactose-beef extract-tryptone broth; suitable dilutions from the pasteurized samples were also tested for living organisms on beef extract-glucose-tryptone agar.

Synopsis of the morphological, physiological and cultural characteristics of the isolated organism[1]

Morphology. Spheres 0·5–0·8 μ in diameter, occurring in chains of variable length, i.e. from 2 to 50 elements or units per chain—majority of chains contained 10–15 units. Stained well. Gram-positive. Non-motile.

Growth in broth. Turbid with fine deposit after 48 hr.

Fermentation reactions. Acid was produced in mannose, glucose, galactose (slight), laevulose, sucrose, maltose, and lactose. No acid in arabinose, xylose, trehalose, raffinose, inulin, glycerol, mannitol, sorbitol and salicin.

Final pH in 1 % lactose broth. $4{\cdot}4 \pm 0{\cdot}1$.

Haemolysis. On human and ox blood agar, colonies appeared as weakly haemolytic or the alpha prime type.

Hydrolysis. (1) Sodium hippurate not hydrolysed. (2) Aesculin not split. (3) Starch not hydrolysed.

Methylene-blue milk. No change was observed.

Growth in litmus milk at different temperatures. 15° C.: no growth. 21° C.: very slight acid development after 15 days. 37° C.: 16 hr. definite acidity; 30 hr. acid coagulation (smooth curd) with incomplete reduction of litmus. 45° C.: 24 hr., acid coagulation and incomplete reduction. 50° C.: very slight growth with weak attempt to produce acidity in some of the cultures.

NH_3 production from peptone. Negative.

Nutrient gelatine. Good growth but no liquefaction.

Beef extract-tryptone-lactose-agar. Four days at 21° C. tiny pin-point colonies. Two days at 37° C. small colonies, circular, greyish white, medium unchanged.

Aerobic, facultative.

Growth in NaCl broth. 2 % NaCl: fair growth. 2·5 % NaCl: slight growth. 3·5 % NaCl: no growth.

[1] For comparative purposes parallel investigations, using cultures of (*a*) a narrow-zoned beta-haem, *Str. agalactiae*; (*b*) *Str. bovis*, a common strain isolated from milk, were also carried out.

Heat resistance

Pasteurization			
Flash	Holder	Plain sterile milk	Nutrient lactose tryptone broth
79·4° C. (175° F.)	—	Survived in large numbers (milk curdled after incubation at 37° C.)	Survived in arge numbers
85° C. (185° F.)	—	Survived, but some cultures failed to curdle milk in incubation at 37° C.	Killed
87·8° C. (190° F.)	—	Killed	—
—	62·8° C. (145° F.) for 30 min.	A small number of organisms resisted this pasteurization and developed colonies on agar plates on incubating at 37° C.	Killed

Other characteristics

As indicated by a faster rate of fermentation, lactose, sucrose, and maltose seemed to have a direct beneficial effect upon the growth of the organism, more so than the mono-saccharides, mannose and glucose.

Bacto-tryptone proved much more satisfactory for the development and growth of the organism than ordinary peptone. Peptone-beef extract agar medium without the addition of an appropriate carbohydrate, such as lactose or sucrose, gave only slight or negligible growth.

Discussion and conclusion

The organism studied had in many respects characteristics closely allied to those of *Str. thermophilus* as described by Orla-Jensen (1) and Sherman & Stark (2). For instance, its sensitiveness to sodium chloride (growth being strongly checked by 2·5 % and entirely inhibited by 3·5 % of this salt) showed a physiological kinship to these bacteria and to *Lactobacillus bulgaricus*. Again, its ability to utilize lactose and sucrose more readily than the constituent monosaccharides is comparable to that of certain strains of *Str. thermophilus* noted by Wright (6). The nature of its source and first appearance, its poor growth on ordinary media, its many negative reactions and its high optimum temperature as well as its strong heat-resistant powers may all be considered as typical of the thermophilus group.

On the other hand, while the organism isolated here fermented maltose strongly, *Str. thermophilus* as described in the literature almost invariably fails to ferment this substance; and on the few occasions that it has done so, the fermentation was only very slight. The action of the organism upon red blood cells, causing weak haemolysis, seemed to differ from *Str. thermophilus*, which is usually described as being completely "indifferent" in blood-agar cultures. Also, while giving slight growth at 50° C., this organism differed from the known strains of *Str. thermophilus* which grow actively at this temperature.

The possibility of its association with *Str. bovis* must now be mentioned. A recent publication by Slanetz (7), attention to which was drawn as this work

was nearing completion, seems to strengthen this opinion. In this paper, the author isolated certain strains, which, apparently, had not been previously described in the literature. The strain to which he has given the name *Str. bovis* var. C, which failed to ferment arabinose, inulin, salicin, and raffinose, appears to agree in many of its characteristics with those of the organism studied here.

However, though the organism did not agree in all its characteristics with *Str. thermophilus* as hitherto described and though a similarity with certain strains of *Str. bovis* has been noted, it is considered that the findings and weight of evidence favour its classification with the *thermophilus* group. It is concluded that the organism encountered is a resistant *Str. thermophilus* strain which has adapted itself to contaminate a churn. As *Str. thermophilus* has been isolated only from milk and milk products, it seems that the churn in this case became infected by those bacteria probably through the medium of a cream inefficiently pasteurized.

Though the isolated organism is probably not a human pathogen, its presence in large numbers—as demonstrated by agar plates, is harmful and undesirable if low bacterial counts are to be maintained and proper control exercised over the ripening and keeping qualities of cream and its products. The effects of efficient pasteurization, at least 190° F. (87·8° C.) in the case of flash pasteurization, must be supplemented by giving due care to utensils, plant and processing equipment in the creamery or cream station. That the churn, improperly sterilized, may be an important factor in the harbouring and spreading of certain heat-resistant bacteria must not be overlooked.

Summary

1. A description is given of a weakly haemolytic organism, which first appeared as a contaminant, in the form of pin-point colonies, in agar plates inoculated with dilutions of a cream which had been subjected to partial processing in a butter churn.

2. Though it resembled in many respects certain strains of *Str. bovis* recently isolated from pasteurized milk in America, it is concluded that the majority of its characteristics showed it to be a resistant strain of *Str. thermophilus* which had adapted itself to contaminate a churn.

3. That the churn was responsible for the spread and maintenance of this heat resistant contaminant is regarded as significant and indicates that efficient pasteurization must be followed by storing and processing in sanitary equipment.

I am indebted to Dr M. Grimes for his help and suggestions in the course of this work.

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249. STUDIES ON THE METHYLENE-BLUE REDUCTION TEST

II. COMPARISON BETWEEN THE OLD AND THE MODIFIED METHODS

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(With 1 Figure)

Introductory

In our previous paper (2) we tried to give the mathematical interpretation of the reduction test as carried out by Wilson's modification (6). Denoting by N_0 the initial number of bacterial cells in 1 ml. of milk at the beginning of the test, and by T the decolorization time in hours, we obtained the following equation expressing the relation between these two variables:

$$N_0 = N_T e^{-kT}. \tag{1}$$

The parameters of this equation are: N_T, the number of cells in 1 ml. of milk at the moment of decolorization of the dye, and k, the average coefficient of multiplication of bacteria calculated for each series of tests from the formula

$$k = \frac{\log N_T - \log N_0}{T \log e}. \tag{2}$$

In regard to the activity of the bacteria we made the following assumptions: (*a*) in a given sample of milk submitted to the test the velocity of multiplication of cells is constant during the logarithmic phase of growth, (*b*) in a given sample of milk during the test the rate at which the hydrogen acceptors are taken up by each cell is constant, (*c*) the ratio of the speed at which the cells take up the hydrogen acceptors to their velocity of multiplication is constant both before and during the test in each sample of milk.

The considerations leading to the simple formula (1) can be developed briefly as follows:

There is a well-known (3, 4) formula of the fermenting capacity of a single cell

$$b' = \frac{Bk'}{N'_{T'} - N'_0}, \tag{3}$$

where b' indicates the amount of substance produced or consumed by a single cell per unit of time, B the total quantity of this substance produced or consumed during the time T' by all cells, k' the coefficient of multiplication according to formula (2), $N'_{T'}$ the number of cells at the end of the experiment,

i.e. after the time T', and N_0' the initial number of cells in the experiment. If formula (3) is applied to the reduction test, its symbols will indicate: B, the total quantity of hydrogen acceptors in the milk consumed at the moment of decolorization, and b', the quantity of hydrogen acceptors used up by a single cell per unit of time. There is no reason to suppose that the bacteria multiplying before the test do not consume hydrogen acceptors. One should, therefore, take into consideration the entire period of time from the original contamination of the milk to the end of the reduction test. Let this contamination be taken as N_0'. It represents the initial inoculum resulting from the invasion of the milk by bacteria, but not from their development in the milk. In comparison with the final number of cells in the reduction test, this inoculum is obviously very small and may be omitted. We get therefore

$$b'=\frac{Bk'}{N'_{T'}} \tag{4}$$

and

$$N'_{T'}=\frac{Bk'}{b'}. \tag{5}$$

The amount of hydrogen acceptors B will be used up at the moment of decolorization; then $N'_{T'}=N_T$. Since by assumption (*c*), k'/b' is constant, k' can be replaced by k and b' by b; k and b denote respectively the coefficient of multiplication and the coefficient of uptake of hydrogen acceptors by a single cell during the test. According to (*c*) the reproductive and metabolic activity of the bacteria may vary, but the ratio of the two remains constant. Equation (5) may therefore be written

$$N_T=\frac{Bk}{b}. \tag{6}$$

It follows that the final number of cells should be constant, although the total bacterial activity in the milk may vary in a given sample before the test, as well as from sample to sample during the test.

On assumption (*a*) the relation

$$N_T=N_0e^{kT} \tag{7}$$

still holds good for each initial number of cells in the reduction test. It follows, therefore, that equation (7) solved for N_0

$$N_0=N_Te^{-kT}, \tag{8}$$

expresses directly the relation between N_0 and T in the reduction test. To verify this equation experimentally, it is enough to estimate the values N_0, N_T and T.

This interpretation of the reduction test as carried out by Wilson's modification has given results showing good conformity between the empirical data and the theoretically expected ones, although our assumptions lead to a certain simplification of the problem. We have stated that: (1) the average

values of N_T were almost constant in all series of samples; (2) the values of k deviate relatively widely; (3) in all samples showing a small value of k the decolorization period was longer, while with larger values of k the decolorization time was invariably shorter; (4) the numerical equations obtained by substituting in formula (1) the average empirical values of N_T and k have given in each series of samples curves conforming very well with the distribution of values representing the experimentally obtained relation between N_0 and T. The variations in the decolorization time for the same initial number of cells are due, therefore, to the velocity of growth of bacteria and their metabolic activity or, in other words, to their total activity. These deviations in about 95% of cases did not exceed definite limits, which can be designated the "zone of decolorization".

It should be mentioned that in cases of fairly rapid decolorization, i.e. up to about 2 hr., a delay was uniformly observed. This delay in relation to the theoretically expected time is due to aeration of the milk before the test. About 5% of samples, however, showed a marked delay in decolorization of the dye owing to abnormally slow activity of the bacteria.

The purpose of the present experiments was to make a comparison between the results of the reduction test as carried out by the old method and by Wilson's modification. Several investigators (1, 5), analysing this modification, have expressed different opinions concerning its practical value. Inasmuch as the essential point of the latter modification is the inversion of the tubes at half hourly intervals, we have analysed only the effect of this inversion. We have adopted, therefore, the Wilson procedure in all details, but two parallel tubes were put up, one of which was inverted and the other not. In this way 180 samples of raw milk were examined. The numbers of cells were estimated microscopically in stained preparations. N_0 was estimated in all samples, N_T in those cases in which the decolorization period was over 1 hr.

EXPERIMENTAL RESULTS

The results of all experiments are presented graphically in Fig. 1. The dots show the relationship between the logarithm of the initial number of cells and the reduction time in hours. The old and the modified methods are represented by circles and solid dots respectively. The circles with the dotted centres denote those samples in which the reduction time was the same by both procedures. The squares represent samples deviating abnormally from the general distribution of values.

The value k was calculated for all milk samples taking over 1 hr. to decolorize in which the value N_T was estimated. As in our previous studies, all abnormally deviating samples (squares in the diagram) show also an abnormally low value for k; in the old method this was below 0·400, in the modified method below 0·600. We have, therefore, eliminated these samples from the data of Table 1 illustrating the distribution of values of N_T and k, and their average values.

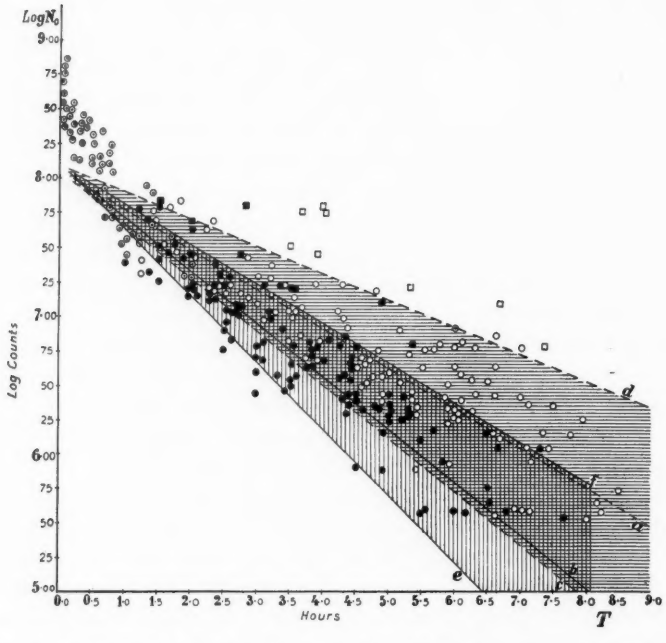

Fig. 1. The "zones" of decolorization: the horizontal shading corresponds to the old method, vertical shading to the modified one. Details in the text.

Table 1

Numbers of cells in 1 ml. of milk at the end of the test: $N_T \times 10^6$						Coefficients of multiplication: k					
		Old method		Modified method				Old method		Modified method	
$N_T \times 10^6$		Frequency	Percentage	Frequency	Percentage	k		Frequency	Percentage	Frequency	Percentage
From	To					From	To				
60	79	4	3·8	1	0·9	0·400	0·499	7	6·6	—	—
80	99	28	26·4	13	12·3	0·500	0·599	27	25·5	—	—
100	119	37	34·9	33	31·1	0·600	0·699	41	38·7	9	8·4
120	139	22	20·8	25	23·6	0·700	0·799	17	16·0	25	23·6
140	159	11	10·4	23	21·7	0·800	0·899	11	10·4	32	30·2
160	179	2	1·9	6	5·7	0·900	0·999	2	1·9	18	17·0
180	199	1	0·9	5	4·7	1·000	1·099	—	—	13	12·3
>200		1	0·9	—	—	1·100	1·199	—	—	6	5·7
Total		106	100	106	100	>1·200		1	0·9	3	2·8
						Total		106	100	106	100

Average value of N_T:
Old method $113{\cdot}4 \times 10^6$
Modified method $126{\cdot}5 \times 10^6$

Average value of k:
Old method 0·660
Modified method 0·885

It is seen that both N_T and k are in the modified method generally larger. More distinct differences are shown by the values of k. Substituting the

average value N_T and k in the formula (1) we obtain the numerical equation for the old method

$$N_0 = 113{\cdot}4 \times 10^6 e^{-0{\cdot}660\,T}, \tag{9}$$

and for Wilson's modification

$$N_0 = 126{\cdot}5 \times 10^6 e^{-0{\cdot}885\,T}. \tag{10}$$

The curves of the above equations are given in the diagram as the lines a and b respectively. They mark out very well the general distribution of the corresponding dots. The distinct shortening of the average reduction time by the modified method seems to be due to a higher rate of bacterial growth in the inverted tube.

The causes of the deviations from the average results of the test were examined in the same way as in our preceding studies. For series of samples showing large and small values of k the average values of N_T and k were calculated. As a large value of k in the old procedure we accepted the value $>0{\cdot}850$, and in the modified method $>1{\cdot}000$; as a small value, k from 0·400 to 0·499 and from 0·600 to 0·699 in the old and the modified methods respectively.

Substituting the corresponding numerical values in the equation (1) we obtain:

	Large values of k	Small values of k
Old method	$116{\cdot}5 \times 10^6 e^{-0{\cdot}896\,T}$	$121{\cdot}6 \times 10^6 e^{-0{\cdot}445\,T}$
Modified method	$134{\cdot}1 \times 10^6 e^{-1{\cdot}115\,T}$	$130{\cdot}6 \times 10^6 e^{-0{\cdot}669\,T}$

(11)

The curves of the equations representing the old and modified methods are given in the diagram as the lines c, d and e, f. It is seen that these curves correspond as accurately as could be expected with the distribution of the extreme dots in both methods. These dots represent the samples showing also extreme values of k. This again corroborates our assumption that the variations in the reduction time are due chiefly to the activity of bacteria, this being measured by the coefficient k. We obtained two "zones of decolorization", and it is evident that the zone of the modified method is significantly narrower than that of the old method.

With regard to the abnormal deviations from the zones of decolorization, the dotted circles represent samples decolorizing in a short time (about 1 hr.), and the squares, samples taking an unusually long time to reduce. The same was observed in our previous studies. Abnormally short reduction times seem to be due to aeration of the milk before the test, unusually long reduction times to sluggish activity of the bacteria. The values of k in the samples denoted by the squares were in the old method from 0·128 to 0·390, and in the modified method from 0·231 to 0·544. On the contrary, the values of N_T do not deviate markedly from those obtained in previous series; the average value of N_T in these samples was in the old method $105{\cdot}6 \times 10^6$, and in the modified method $120{\cdot}1 \times 10^6$.

Before discussing these findings, we give Table 2, illustrating the general numerical results of the comparison between both methods. The table gives

the limits of variation in the number of bacterial cells for the given period of decolorization. These numerical limits, being calculated according to formula (11), correspond to the "zones" illustrated in the diagram. It should be emphasized that by both methods the number of samples exceeding these limits is the same, thus ensuring a fair comparison of the two.

Table 2. *Numbers of cells corresponding to the time of reduction*

Time of decolorization in hours	Numbers of cells in millions per 1 ml.			
	Old method		Modified method	
	From	To	From	To
8	0·09	3·5	0·02	0·6
7	0·2	5·4	0·05	1·2
6	0·5	8·4	0·2	2·4
5	1·3	13·1	0·5	4·6
4	3·2	20·5	1·6	9·0
3	7·9	32·0	4·7	17·6
2	19·4	49·9	14·4	34·2
1	47·6	77·9	44·0	66·8

It is seen that in Wilson's modification the variations in the numbers of bacteria corresponding to the same period of decolorization are much smaller, and that the time of reduction, especially with the better quality milks, is significantly shortened.

The shortening of the time of reduction in the modified method is readily appreciated from the following figures:

With a reduction time in hours by the old method of							
8	7	6	5	4	3	2	1
the period of decolorization is in the modified method shorter on the average by							
1 hr. 55 min.	1 hr. 40 min.	1 hr. 24 min.	1 hr. 09 min.	54 min.	38 min.	23 min.	8 min.

The smaller variation in the number of bacteria corresponding to a given time of reduction, and the shortening of the reduction time itself, are both recommendations in favour of the modification suggested by Wilson. The contamination of the milk is by this method more accurately appreciated; the results of the test are more regular; and, at the same time, the experimental error resulting from keeping the micro-organisms in the layer of fat, and eventually also from their clumping, is in fact eliminated.

Discussion

We should first like to draw attention to the close conformity, on the average, between the experimental and the theoretical data. As in our previous studies, the curves of the numerical equations in all cases correspond with the empirical data from which they were elaborated. This conformity can be regarded as justifying our theoretical interpretation of the reduction test. Inasmuch as this interpretation was fully discussed in the first part of our studies, it will be sufficient to make here a few remarks only.

The activity of the bacterial cells in the milk seems to be responsible for the differences in the velocity of the reduction process observed with the same initial number of cells tested by the same method, and for the differences in the reduction time between the two methods. By "activity" we denote two distinct functions of the cells, namely their rate of growth and their consumption of hydrogen acceptors. We have assumed that these two functions bear a more or less constant relationship to each other. The approximate constancy of the value N_T corroborates this assumption (see equation (6)). In particular series of samples we have obtained the average values of N_T per 1 ml. of milk:

By the old method	$113{\cdot}4 \times 10^6$	$116{\cdot}5 \times 10^6$	$121{\cdot}6 \times 10^6$	$105{\cdot}6 \times 10^6$
By the modified method	$126{\cdot}5 \times 10^6$	$134{\cdot}1 \times 10^6$	$130{\cdot}6 \times 10^6$	$120{\cdot}1 \times 10^6$

These values show a certain amount of variation, but are on the whole very constant. As we see, the values of N_T are in the modified method somewhat larger. Probably the greater increase of bacteria is due to inversion of the tubes, which causes some aeration of the milk. This aeration, however, does not disturb the course of the test, although some disturbance would have been expected theoretically as the result of variations in the value of B, i.e. of the total quantity of hydrogen acceptors in the milk.

Our experiments entirely corroborate Wilson's surmise, that the inversion of the tubes stimulates bacterial growth. The average coefficient of multiplication is in the modified method equal to 0·885 and in the old method to 0·660.

The coefficients of multiplication of bacteria k were calculated from formula (2), i.e. on the assumption that during the test the bacteria are in the logarithmic phase of growth. Such an assumption is obviously a simplification of the true rate of growth, which undoubtedly is more complicated. The value of k must be treated only as an average indicating whether the bacteria are growing more slowly or more quickly in a given sample. Owing to the direct proportionality between the rate of growth and the intensity of respiration of the cells, the value k measures the average total activity of the bacteria. Thus, it makes no difference to the final result of the test, i.e. the time of reduction, whether the bacteria were initially in the lag phase and then grew rapidly in the logarithmic phase, or whether they were initially in the logarithmic phase, but grew all the time more slowly. It should be emphasized that the average values of k and N_T actually obtained in the modified method do not differ from those obtained in our previous studies ($k = 0{\cdot}871$, $N_T = 125{\cdot}2 \times 10^6$).

SUMMARY

In order to compare the old and the modified methods of the methylene-blue reduction test, 185 samples of raw milk were examined. We followed Wilson's method absolutely, but included two tubes in each test, one of which was inverted and the other not.

The results obtained and their interpretation agreed with the theoretical

expectations given in our previous paper(2), and briefly outlined at the beginning of the present paper.

It is found that the deviations in the reduction time, corresponding to a given initial number of cells, are smaller by the modified than by the old method, and that the average time of reduction is shorter.

The smaller variation in the reduction time corresponding to a given number of cells at the beginning of the test is due probably to a more uniform distribution of the bacteria in the milk following the inversion of the tube. Wilson's suggestion that the shortening of the reduction time in the modified method is due to the stimulation of bacterial growth proves to be entirely correct. We obtained the average coefficients of multiplication in the old method of 0·660, and in the modified of 0·885.

Our results confirm on the whole the positive value of the modification of the methylene-blue reduction test as suggested and worked out by Wilson.

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576.85 str.:577.15.062

250. THE INFLUENCE OF VARIOUS FACTORS ON THE FERMENTATION END-PRODUCTS OF THE HETERO-FERMENTATIVE STREPTOCOCCI

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(With 1 Figure)

THE four factors studied in this work (temperature, *p*H, oxygen tension and the presence of growth factors) are those most likely to vary in the ripening of dairy products, and the by-products investigated (acetic acid and alcohol), if not themselves immediately responsible for the typical flavours in cheese and other dairy products, may serve as a useful index of potential flavour substances. The importance of temperature is well recognized. Thus, for example, it is generally accepted that lower ripening temperatures result in better flavoured cheese. The significance of the growth factor effect lies in the obvious fact that in practice lactic acid bacteria never grow in pure culture, but in association with other micro-organisms many of which produce growth factors for the more fastidious heterofermentative types (1). Moreover the rate of growth appears to be correlated with the proportion of by-products (2). In these experiments this "growth factor effect" has, for controlled experimental purposes, been simulated by the addition of yeast autolysate.

Perhaps the greatest interest of the present work lies in the "aeration effect", a subject of much speculation but little experimental evidence. Lactic acid bacteria are peculiar in producing mainly lactic acid, even in the presence of air. Other types of organisms which may produce a pure lactic acid fermentation anaerobically, form only traces of lactic acid in air (3). The Pasteur effect, or the suppression of the true lactic acid fermentation by respiration, should result in an increased production of by-products. The quantitative effect of such a mechanism is thus a matter of some economic importance, especially for the heterofermentative group.

Various workers (Orla-Jensen (4), Hucker & Pederson (5), Hammer (6) and Davis (7,8)), have been interested in the classification of this group, which includes the genera *Betacoccus* (Orla-Jensen) and *Leuconostoc* (van Tieghem, amended by Hucker) and the two species *Str. citrovorus* and *Str. paracitrovorus* of Hammer. Peterson, Fred & Anderson (9) have studied the effect of the carbon source on the ratios of by-products formed.

Twelve strains of streptococci were used in the present experiments. Eight were strains of various types of heterofermentative streptococci. For com-

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parison strains of *Str. cremoris*, *Str. faecalis* and *Str. diaceti-lactis* were used. The streptococci examined were selected from the stock cultures maintained at this Institute by Dr J. G. Davis whose classification of these strains (7, 8) has been adopted here. Table 1 gives their classification and original sources.

Table 1. *Sources of strains and classification*

Name	Code name	Name under which received	Source
Str. citrovorus	S. 32	*Str. citrovorus*	B. W. Hammer (Ames, Iowa)
	S. 146	*Str. citrovorus*	B. W. Hammer (Ames, Iowa)
Str. mesenterioides	3274	*Leuc. mesenterioides*	National Collection of Type Cultures, Lister Institute (London)
	X. 5	*Leuc. mesenterioides*	C. S. Pederson (Geneva, U.S.A.)
Str. dextranicus	D. 22	*Str. paracitrovorus*	B. W. Hammer (Ames, Iowa)
	DN. 2	*Str. paracitrovorus*	B. W. Hammer (Ames, Iowa)
	X. 22	*Leuc. dextranicum*	C. S. Pederson (Geneva, U.S.A.)
	Bc. bovis	*Bc. bovis*	S. Orla-Jensen (Copenhagen)
Str. diaceti-lactis	6	*Str. diaceti-lactis*	T. Matuszewski (Warsaw)
	15	*Str. diaceti-lactis*	T. Matuszewski (Warsaw)
Str. faecalis	O.J.	*Str. faecium*	S. Orla-Jensen (Copenhagen)
Str. cremoris	H.P.	*Str. cremoris*	H. R. Whitehead (Palmerston North, N.Z.)

The influence of the four factors mentioned on the final concentrations of lactic and acetic acids and alcohol after exhaustive growth were investigated by the following methods, separated milk being used as the basal medium:

(*a*) The influence of temperature was studied by growing the organisms at temperatures as far below and above the optimum as was consistent with reasonable growth. (*b*) Aerobic and anaerobic conditions were compared by culturing in conical flasks with a shallow layer of medium for the aerobic cultures and in small round flasks filled to the neck for anaerobic cultures. (*c*) The influence of *p*H could not be investigated fully but the addition of chalk ensured that the *p*H did not fall as rapidly as in cultures to which no chalk was added. (*d*) Yeast autolysate was added to the basal medium to enable the influence of "growth factors" to be investigated. Yeast was used as it has been shown to be generally superior to other plant extracts as a source of "growth factors" (10).

The principal compounds produced by lactic acid bacteria from sugar are lactic acid, acetic acid, ethyl alcohol and carbon dioxide. With such a large number of cultures it was found impossible to estimate the carbon dioxide, and analyses were confined to the other three compounds. In addition Voges-Proskauer tests for acetyl-methyl-carbinol and diacetyl were carried out regularly, and the values for amino nitrogen were determined by Sørensen titrations. The final *p*H values were determined by the quinhydrone electrode.

Methods

Media. The media used were separated milk and separated milk plus 5% yeast autolysate (6 days' autolysis (11)). The milk was drawn from a bulk obtained from disease-free animals. In order to ensure uniform composition of

media in each flask the milk was sterilized in bulk. Four litres of the medium were placed in a 5 l. round flask and after removing the false bottom from the steam sterilizer the flask was placed in the water (already boiling). Additional burners were used under the steamer and by this method it was found that the deeper parts of the medium were heated to 98° C. in about 20 min., so that by heating on each of three consecutive days for 40 min. after placing the flask in the steamer, followed by immediate cooling in running water, effective sterilization without appreciable discoloration was obtained. The hot medium was siphoned aseptically into 1 l. flasks to a depth of 2 cm. for the aerobic cultures and into 250 ml. round flasks, which were filled to the neck, for anaerobic cultures. About 9 g. of sterile chalk (previously heated in test tubes to 185° C. for 9 hr.) were added, when required, to the flask before running in the medium. A 2 cm. layer of sterile paraffin oil was added to all anaerobic cultures.

After weighing, all flasks of media were incubated for 3 days at 37° C. followed by 4 days at room temperature to test for sterility and then inoculated with 1 ml. of a freshly clotted yeast-beer-wort-dextrose-litmus-milk culture of the test organism. The flasks were covered with a double thickness of greaseproof paper secured by a heavy rubber band. During incubation all aerobic cultures, and anaerobic cultures containing chalk, were vigorously agitated twice daily. Two incubation temperatures were used for each strain; the lower temperature was in all cases 19° C. (8 weeks' incubation), and the higher temperature 32° C. except for strains 3274, X. 5, X. 22, *Bc. bovis*, and *Str. faecalis* where it was 37° C. (4 weeks' incubation).

Preparation of cultures for analysis. After weighing, 1 ml. of liquid was removed aseptically from all flasks including the control and inoculated into Lemco broth and yeast dextrose litmus milk, and the purity of these cultures ascertained by microscopic examination, the catalase test, and observation of type of growth. Evaporation losses from the flasks were then made up with water, clotted cultures reduced to an even consistency by means of a household egg-beater and chalk removed by light centrifuging. To prevent decomposition. 10 ml. of chloroform were added to each flask.

Most of the chemical determinations were carried out on a protein free filtrate obtained as follows: 20 ml. of the control medium was weighed into a 110 ml. graduated flask with an accuracy of 0·01 g. The same weight of culture was taken from each of the other cultures in the series. After diluting to 95 ml. with water, 5% phosphotungstic acid in 5% sulphuric acid was added to the graduation mark, and the precipitate removed by centrifuging.

Analytical methods. Analyses for sugar, lactic acid and alcohol were carried out on the protein-free filtrates, determinations being made in duplicate.

Sugar. The chloramine-T method of Hinton & Macara (12) was used. As some bacteria hydrolyse excess lactose in the medium, it was necessary to carry out further determinations after acid hydrolysis and it was found that heating at 100° C. for 30 min. with a final concentration of 1·5*N* hydrochloric

acid gave a 97–98% hydrolysis. Where comparison of the figures before and after acid hydrolysis indicated lactase activity the total hexose figure, corrected for incomplete hydrolysis of the lactose, was used to calculate residual sugar. The appropriate correction factor, which varied slightly in each series, was calculated from the figures given by the control medium.

Lactic acid. The method of Friedemann & Kendall(13) was used, the acetaldehyde being removed by aeration. The incoming air was passed through bisulphite solution to guard against errors from absorption of formalin vapour.

Acetic acid. All the steam volatile acid was assumed to be acetic acid. Fifty ml. of culture were acidified to *p*H 2 with 10% sulphuric acid and the volume made up to 100 ml. Distillations were carried out in the apparatus described by Hiscox & Harrison(14), 5 vol. of distillate being collected. No corrections for carbon dioxide or the steam volatility of lactic acid were made.

Alcohol. Widmark's(15) method and the apparatus of Meyerhof and Kiessling(16) were used. Volatile aldehydes and ketones were first precipitated with 2-4-dinitrophenylhydrazine in acid solution in the distillation flask, the mixture approximately neutralized with NaOH and the *p*H adjusted to about 7·2, to prevent distillation of volatile acids, by adding solid disodium hydrogen phosphate. Incomplete distillation of the alcohol occurred unless the rate of flow of carbon dioxide was 90–100 ml. per minute, and it was found necessary to control this rate by means of Venturi meters.

The Sørensen titrations and *p*H determinations were carried out on the original material, and Voges-Proskauer tests on 2 ml. of distillate from the distillation of 30 ml. of the protein-free serum, to which a few drops of acid ferric chloride had been added.

Discussion

The analytical results are given in Table 2. The total production of end-products and sugar utilizations (as hexose anhydride) are stated in millimols per cent, and from these figures the ratios of equivalents of end-products to equivalents of hexose used and lactic acid produced have been calculated. Thus it is possible to assess the influence of the various factors on the ratios of end-products as well as the absolute amounts.

The percentages of the sugar used accounted for by the total end-products recovered is also given in Table 2. The percentage recovery is very variable, and since sugar determinations in such a complex medium are subject to considerable error it was felt that the ratios of alcohol and acetic acid to lactic acid formed would provide a sounder basis for comparison than the ratios to carbohydrate disappearing from the medium. Both ratios are given in Table 2, but inspection shows that the same conclusions result whichever ratio is considered.

Total growth. With the heterofermentative types little or no growth occurred unless yeast was added, but with the homofermentative types the

addition of yeast did not greatly increase the total amount of sugar utilized.

Lactic acid. The total production of lactic acid in cultures without chalk is increased by anaerobic conditions and low temperature. Considering the ratio of mM. of lactic acid produced to mM. of hexose utilized it may be seen that comparatively more lactic acid is produced anaerobically, and in the presence of chalk, but temperature and the addition of yeast have no significant influence.

These conclusions regarding lactic acid production apply to both the heterofermentative and homofermentative streptococci, but as the latter produce little or no acetic acid and no alcohol the following sections refer to the heterofermentative types only.

Acetic acid. The total amount of acetic acid is higher in the presence of chalk, at the lower temperature and in yeast milk, but is decreased by anaerobic conditions. The ratio of acetic acid, however, to both sugar utilized and lactic acid produced is smaller at the lower temperature, in the presence of chalk and "growth factors" and under anaerobic conditions. The fact that the ratio of acetic acid to sugar utilized decreased at the lower temperature is contrary to the findings of Orla-Jensen (4), only one strain of *Str. dextranicus* (X. 22) proving an exception in this respect. Orla-Jensen (4) also found that the relative amount of acetic acid was about the same both in the presence and absence of chalk.

Alcohol. The total alcohol production is higher when yeast or chalk is added to the medium, under anaerobic conditions and at the lower temperature, and similarly the ratio of alcohol found to sugar utilized and lactic acid produced, is increased. The weight of alcohol is always far greater than that of acetic acid, and it may be noted that the presence in heterofermentative and the absence in homofermentative streptococci of enzymes permitting alcoholic fermentation is the most striking single difference between the two types of bacteria. Carbon dioxide was not estimated, but assuming that the mechanism of its production in these organisms is the same as in the yeast the total production and ratios to sugar used and lactic acid formed would vary in exactly the same way as the alcohol.

In Table 3 the ratio of by-products (acetic acid and alcohol) to total end products (by-products plus lactic acid) is given as a percentage. With the heterofermentative types no marked species or strain difference is shown and, on a weight basis, by-products form 30–40% of the total end-products. With the homofermentative types only traces of by-products are formed but the findings with the two strains of *Str. diaceti-lactis* are particularly interesting. Strain 6 has been maintained in this laboratory for several years but strain 15 was a fresh isolation received from Professor Matuszewski. The older strain had lost its power to form diacetyl in milk and produced no alcohol and practically no acetic acid, whereas the freshly isolated strain produced diacetyl (Voges-Proskauer test) and a considerable quantity of acetic acid but no

Table 3. *Ratio of by-products to total end-products* (%)

$$\frac{(\text{Acetic acid} + \text{alcohol}) \times 100}{(\text{Lactic acid} + \text{acetic acid} + \text{alcohol})} \text{ (weight basis)}$$

Strain		Ae.	Ae.C.	An.	An.C.
Str. citrovorus: S. 32	S. 32	—	—	—	—
	S. 19	—	—	—	—
	Y. 32	41	34	37	37
	Y. 19	43	36	37	35
S. 146	S. 32	—	—	—	—
	S. 19	—	—	—	—
	Y. 32	37	36	36	36
	Y. 19	40	36	39	39
Str. mesenterioides: 3274	S. 37	—	—	—	—
	S. 19	—	—	—	—
	Y. 37	32	33	32	36
	Y. 19	48	38	36	34
X. 5	S. 37	—	—	—	—
	S. 19	42	36	35	31
	Y. 37	42	36	39	34
	Y. 19	40	42	33	34
Str. dextranicus: D. 22	S. 32	61	48	52	45
	S. 19	24	27	25	27
	Y. 32	26	36	43	34
	Y. 19	40	35	43	36
DN. 2	S. 32	64	47	40	40
	S. 19	43	39	39	39
	Y. 32	41	37	49	35
	Y. 19	47	41	46	38
X. 22	S. 37	45	35	42	41
	S. 19	45	47	47	46
	Y. 37	38	33	43	38
	Y. 19	44	39	38	39
Bc. bovis	S. 37	—	—	—	—
	S. 19	—	—	—	—
	Y. 37	36	37	—	38
	Y. 19	45	39	38	40
Str. diaceti-lactis: 15	S. 32	14	7	1	6
	S. 19	1	6	11	6
	Y. 32	10	5	11	4
	Y. 19	8	4	7	2
6	S. 32	1	1	1	1
	S. 19	1	1	1	1
	Y. 32	2	2	1	2
	Y. 19	3	2	1	2
Str. faecalis: O.J.	S. 37	1	1	1	1
	S. 19	1	1	1	1
	Y. 37	1	1	—	1
	Y. 19	1	6	1	1
Str. cremoris: H.P.	S. 32	0	—	0	0
	S. 19	3	1	2	1
	Y. 32	0	0	0	0
	Y. 19	1	2	2	1

Ae. = aerobic; C. = chalk; An. = anaerobic; 19, 32, 37 = ° C.;
S. = separated milk; Y. = yeast separated milk.

alcohol. It seems that after long cultivation in the laboratory *Str. diaceti-lactis* reverts to a form closely resembling *Str. lactis*, and moreover as it is only slightly stimulated by the addition of yeast to milk it cannot be classified with the true heterofermentative types. The only other strain studied that

produced diacetyl under the conditions of these experiments was *Str. faecalis* and it seems that *Str. diaceti-lactis* is more closely related to it than to the heterofermentative bacteria. The absence of the alcoholic fermentation in both cases strengthens this contention.

In Fig. 1 the data obtained with *Str. citrovorus* (S. 146) are given in graph form. As no significant differences were found between the species, these results are typical for the heterofermentative streptococci and clearly show the effects of the factors investigated on the total production of end-products and the ratios of the various end-products formed to the sugar utilized.

Final pH values. These values are given in Table 4, from which it may be seen that for the heterofermentative types the lowest *p*H figures are obtained in the yeast milk at the lower temperature. The lowest *p*H values ranged from 4·1 (X. 22) to 4·75 (X. 5, DN. 2). With the homofermentative strains no marked differences in *p*H were found between the separated milk and the yeast-separated-milk cultures, but the lowest *p*H values were again found at the lower temperature (range *p*H 4·0–4·2). Many of the cultures to which chalk had been added were clotted even though they were shaken twice daily from the time of inoculation. Chalk is unsatisfactory for controlling the *p*H and in many cases values below 5·5 were found. The *p*H values of the controls were lower after incubation for 4 weeks at 32 or 37° C. than after incubation for 8 weeks at 19° C. The maximum difference recorded is 0·14 of a *p*H unit.

Sørensen nitrogen titrations. No significant change in the Sørensen titration figures occurred except in the case of *Str. diaceti-lactis* (Strain 6) where a decided increase was noted in the cultures containing chalk.

Lactase activity. Hydrolysis of the residual lactose occurred in several instances. With both strains of *Str. citrovorus*, even in the plain milk cultures where growth was very limited, considerable hydrolysis had taken place and in the yeast milk cultures practically all the residual lactose had been hydrolysed. In general it may be seen from Table 5 that lactase activity is most marked where extensive growths had taken place, as in the chalk cultures, and in cultures with yeast. This is in agreement with the theory that lactase is only liberated from the cell after death (4).

General. A feature of the data presented is the relative inability of the respiratory enzyme systems to suppress fermentation under aerobic conditions. Although the Pasteur effect is not found in the non-respiring homofermentative lactic acid bacteria (17) it seems surprising that it is almost absent in the heterofermentative streptococci which possess respiratory enzyme systems. As Davis & Rogers (8) have shown, cell suspensions of the heterofermentative streptococci take up oxygen rapidly, but under the present cultural conditions alcoholic fermentation proceeded almost as well under aerobic as under anaerobic conditions and acetic acid production was not greatly stimulated by the presence of free oxygen. As acetic acid production was almost as marked anaerobically as aerobically its respiratory origin is doubtful.

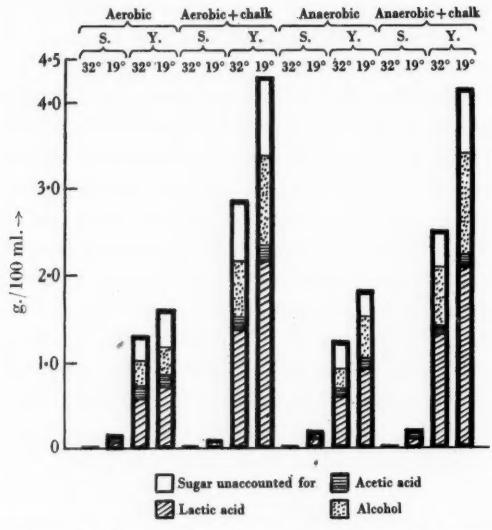

Fig. 1A. Sugar used and end-products formed. Organism: *Str. citrovorus* (S. 146).
S. = Skim milk. Y. = Yeast skim milk.

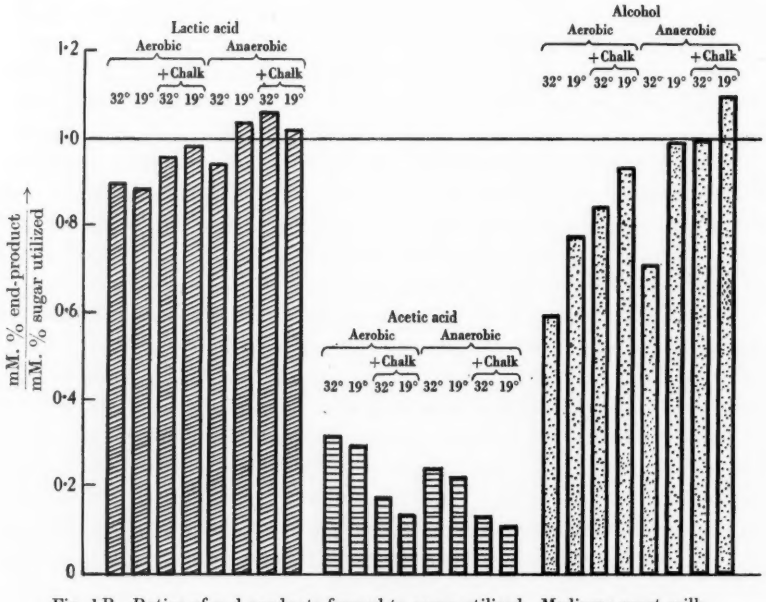

Fig. 1B. Ratios of end-products formed to sugar utilized. Medium: yeast milk.
Organism: *Str. citrovorus* (S. 146).

Table 4. *Final* pH *values*

Strain		Control	Ae.	Ae.C.	An.	An.C.
Str. citrovorus: S. 32	S. 32	6·31	6·13	7·03	6·07	6·38
	S. 19	6·31	6·03	6·76	5·80	6·27
	Y. 32	6·40	4·99	6·04	5·02	5·85
	Y. 19	6·40	4·74	6·06	4·67	5·48
S. 146	S. 32	6·29	6·19	7·10	6·21	6·69
	S. 19	6·29	6·10	6·77	6·02	6·35
	Y. 32	6·23	4·68	5·90	4·54	5·82
	Y. 19	6·36	4·59	5·95	4·24	5·47
Str. mesenterioides: 3274	S. 37	6·39	6·23	5·86	6·29	6·70
	S. 19	6·37	6·20	6·94	6·25	6·65
	Y. 37	6·29	5·05	5·86	4·82	5·87
	Y. 19	6·40	4·45	5·82	4·57	5·42
X. 5	S. 37	6·34	6·18	6·50	5·83	6·40
	S. 19	6·32	5·59	5·91	5·64	5·45
	Y. 37	6·15	4·96	5·55	5·30	5·05
	Y. 19	6·35	4·75	5·55	4·91	5·18
Str. dextranicus: D. 22	S. 32	6·31	5·57	6·06	5·47	5·88
	S. 19	6·45	5·25	5·90	5·69	5·76
	Y. 32	6·31	4·79	5·39	5·08	5·55
	Y. 19	6·46	4·63	5·35	4·94	5·17
DN. 2	S. 32	6·25	5·40	6·14	5·61	6·09
	S. 19	6·26	5·16	5·75	5·25	5·78
	Y. 32	6·33	4·82	5·42	5·47	5·47
	Y. 19	6·37	4·75	5·43	4·91	5·29
X. 22	S. 37	6·45	5·64	6·00	5·78	6·07
	S. 19	6·48	5·52	6·05	5·57	5·95
	Y. 37	6·38	4·54	5·51	4·81	5·75
	Y. 19	6·48	4·10	5·58	4·30	5·53
Bc. bovis	S. 37	6·30	6·14	7·00	6·16	6·66
	S. 19	—	—	—	—	—
	Y. 37	6·20	4·92	5·57	—	5·30
	Y. 19	6·37	4·41	5·50	4·22	5·49
Str. diaceti-lactis: 15	S. 32	6·35	4·56	5·38	4·56	5·55
	S. 19	6·35	4·37	5·60	4·41	5·28
	Y. 32	6·34	4·40	5·37	4·27	5·41
	Y. 19	6·40	4·12	5·39	4·12	5·25
6	S. 32	6·29	4·52	5·13	4·49	5·20
	S. 19	6·40	4·51	5·16	4·41	5·05
	Y. 32	—	5·10	5·44	5·02	5·45
	Y. 19	6·36	4·23	5·23	4·37	5·12
Str. faecalis: O.J.	S. 37	6·22	4·52	5·57	4·55	5·70
	S. 19	6·35	4·60	5·65	4·35	5·16
	Y. 37	6·27	4·36	5·15	—	5·25
	Y. 19	6·46	4·20	5·36	4·12	4·98
Str. cremoris: H.P.	S. 32	6·26	4·23	—	4·13	4·19
	S. 19	6·26	4·00	5·31	4·06	5·12
	Y. 32	—	4·37	4·27	4·25	5·30
	Y. 19	6·21	4·20	4·70	4·17	4·80

Ae. = aerobic; 19, 32, 37 = ° C.; An. = anaerobic; C. = chalk;
S. = separated milk; Y = yeast separated milk.

Table 5. *Percentage of residual lactose hydrolysed*

Strain		Ae.	Ae.C.	An.	An.C.
Str. citrovorus: S. 32	S. 32	21	15	26	21
	S. 19	7	10	9	12
	Y. 32	35	93	29	85
	Y. 19	23	91	28	64
S. 146	S. 32	30	31	28	40
	S. 19	18	13	22	18
	Y. 32	48	86	42	73
	Y. 19	53	73	48	50
Str. mesenterioides: 3274	S. 37	0	0	0	8
	S. 19	0	0	0	0
	Y. 37	0	5	0	6
	Y. 19	0	28	0	55
X. 5	S. 37	0	0	0	0
	S. 19	0	0	0	0
	Y. 37	29	31	25	32
	Y. 19	0	24	0	17
Str. dextranicus: D. 22	S. 32	0	0	0	0
	S. 19	0	0	0	0
	Y. 32	0	0	0	0
	Y. 19	0	12	0	10
DN. 2	S. 32	0	0	0	0
	S. 19	0	0	0	0
	Y. 32	0	0	0	0
	Y. 19	0	35	0	14
X. 22	S. 37	0	8	0	0
	S. 19	0	0	0	0
	Y. 37	0	6	0	0
	Y. 19	0	25	0	70
Bc. bovis	S. 37	0	0	0	0
	S. 19	0	0	0	9
	Y. 37	0	13	0	12
	Y. 19	0	16	0	63
Str. diaceti-lactis: 6	S. 32	0	0	0	0
	S. 19	0	15	0	0
	Y. 32	0	0	0	0
	Y. 19	0	47	0	7
Str. faecalis: O.J.	S. 37	0	0	0	0
	S. 19	0	0	0	0
	Y. 37	0	0	0	0
	Y. 19	0	24	0	13

Ae. = aerobic; An. = anaerobic; 19, 32, 37 = ° C.; C. = chalk; S. = separated milk; Y. = yeast separated milk.

Summary

1. The influence of temperature, *p*H, oxygen tension and yeast autolysate on the production of by-products and the ratios of by-products formed to sugar utilized and lactic acid produced in milk by the heterofermentative lactic acid streptococci has been investigated.

2. The total production of lactic acid is increased by anaerobic conditions and low temperature. The ratio of lactic acid formed to sugar utilized is increased by anaerobic conditions and the presence of chalk.

3. The total amount of acetic acid is higher in the presence of chalk, at the lower temperatures and in yeast milk, but is decreased by anaerobic

conditions. The ratio of acetic acid both to sugar utilized and to lactic acid formed is smaller at lower temperatures, in the presence of "growth factors" and chalk and under anaerobic conditions.

4. The total alcohol production is higher when yeast or chalk is added to milk, under anaerobic conditions and at lower temperatures, and similarly the ratio of alcohol formed to sugar utilized and lactic acid produced is increased.

5. Hydrolysis of residual lactose occurred in all cultures of *Str. citrovorus* and with some of the other streptococci in cultures to which chalk had been added.

6. Although the heterofermentative lactic acid streptococci possess respiratory enzymes, these fail to suppress aerobic fermentation (i.e. the Pasteur effect is slight).

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251. THE ACTION OF CHEMICAL DISINFECTANTS ON BACTERIOPHAGES FOR THE LACTIC STREPTOCOCCI

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The widespread occurrence in New Zealand cheese factories of phages active against the organisms in starter cultures made it desirable to investigate means for disinfection so that the possibilities of contamination of starter cultures with phage could be minimized. Cheese whey commonly contains a high concentration of phage (1), and since this whey comes into contact with all the utensils and splashes over much of the wall surface of the factory it is inevitable that phage should be widespread. It has previously been reported (2) that phages for the lactic streptococci are destroyed by a heat treatment at 70–75° C. for 30 min. at *p*H 6·0. It is thus quite possible to destroy phage in whey by heat, but even then there would remain large surfaces on vats, floors and walls where splashes of unsterilized whey might dry and provide sources of phage infection. It was decided therefore to make trials of the phage-destroying powers of the various chemical disinfectants to see whether there was any one of them which, in the form of a rinse or a spray, could be used for disinfecting the surroundings of the starter cultures in a dairy factory.

The essential features of any disinfectant to be used in this connexion are (*a*) absence of deleterious influence on dairy produce and absence of poisonous effect on animals and human beings, and (*b*) rapidity of action. The second feature is important because with the development of phage fairly regularly in cheese vats there is a constant reinfection of the surroundings. One can hope only to rid the starter surroundings of phage for an hour or so while subculturing processes are carried out.

There have been many investigations in the past on the action of chemicals on bacteriophage but such a variety of techniques and such a variety of phage races have been used that, as is pointed out by Krueger in his excellent review of the subject (3), comparison of the results is almost impossible. There is no detailed information whatever about the action of chemicals on phages for the lactic streptococci.

The experiments to be described were not designed to elucidate the mechanism or the dynamics of the destruction of the phages by chemicals. They were directed toward the discovery of a means for destroying completely in a very short time a high concentration of phage in a specific medium (cheese whey). Disinfection experiments on phage differ from those on

bacteria in that the bacteria can, if it is so desired, be washed free from growth medium and suspended in water or saline. Phage, on the other hand, due to the mechanism of its development on sensitive organisms, is necessarily always associated (in the absence of very laborious purifying procedures) with large amounts of proteins from the medium in which the organisms were grown. These inert constituents obviously modify the influence of chemicals on the phage. A protective action is to be expected. In the present instance the phage preparations contained on the average 0·67 % protein and 4·41 % lactose. Probably most of the chemicals used would have been effective in lower concentrations or in shorter times had these proteins and carbohydrate been absent.

METHODS

Eight representative phages were used in the experiments. The characteristics of the phages have been described in a previous paper (4). For the present experiments they were all propagated on the susceptible organisms growing in samples of the one batch of milk. In spite of this there were variations in the protein, lactose and *p*H values of the different preparations. The significance of these variations in so far as they affected phage destruction will be dealt with later. The phages were all of the same order of strength, giving titres of 10^{-7}–10^{-8}.

The experiments were carried out at room temperature (15–16° C.). Equal volumes of phage and chemical solution (of various strengths) were mixed in sterile tubes and, after the elapse of a specified time interval, a standard loopful of the reaction mixture was transferred to sterilized milk inoculated with the susceptible organism. In this way any surviving phage could develop on the susceptible organism. In each experiment the volume of milk was adjusted so that the amount of disinfectant transferred on the loop was not present in sufficient concentration to interfere with growth. The inoculated milk was incubated for about 5 hr. at 37° C. then overnight at 22° C. The failure of a culture to coagulate indicated the presence of surviving phage. Clotted cultures were subcultured three times in order that any trace of phage might have the opportunity to develop sufficiently to lyse the organisms. The method adopted constituted therefore a rigorous test for complete destruction of the phage. Appropriate control tubes containing phage alone and organisms alone were included in each experiment.

EFFECT OF H^+ AND OH^- IONS

Our phage preparations all varied slightly in reaction and it was thus important to know what effect, if any, acids and alkalies had on their stability. Rough experiments showed that addition of lactic acid to a phage preparation to a final concentration of 2·5 % destroyed the phage at room temperature in less than 5 min. A concentration of 1 % lactic acid was effective in less than 24 hr. The inactivation of the phage in 2·5 % lactic acid was not reversible on neutralization of the acid after 15 min. so it appeared that a true destruction

was brought about. Mineral acids had a similar effect, also high concentrations of alkali.

More detailed experiments showed that destruction was rapid at room temperature at and beyond the limiting *p*H values of 2·5 and 11·8. Between these values the destructive effect fell off rapidly, and in particular between *p*H 4·0 and 7·0 there was no appreciable effect over a period of five days. Our phage preparations had *p*H values between 5·23 and 6·11 (average value 5·84); thus it was evident that the concentration of H^+ ions would not significantly affect results obtained with disinfectants in experiments extending over a few days.

Results obtained with several chemical disinfectants

The results obtained with seven substances acting on phage race hp. are given in Table I. Only some of the disinfectants are suitable for use in dairy factories. The others were included for purposes of comparison.

Table I. *Power of disinfectants in destroying phage*

Disinfectant	Final concentration in phage-disinfectant mixture %	Time required for complete destruction
Hypochlorite (available chlorine)	0·05	Less than 1 min.
$KMnO_4$	0·25	Less than 1 min.
	0·05	Between 1 and 5 min.
	0·025	Not in 2 days
H_2O_2	3·0	Between 15 and 60 min.
	2·5	Between 1 and 24 hr.
	0·5	Between 1 and 24 hr.
HCHO	5·0	Between 5 and 30 min.
	2·5	Between 30 and 60 min.
	1·0	Between 1 and 24 hr.
	0·5	Between 1 and 24 hr.
$HgCl_2$	2·5	Between 1 and 24 hr.
	1·0	Between 2 and 3 days
	0·5	Not in 14 days
Alcohol	90·0	Between 3 and 4 days
	85·0	Between 2 and 3 days
	80·0	Between 2 and 3 days
	75·0	Between 3 and 4 days
	70·0	Not in 6 days
Phenol	2·5	Not in 14 days

The most striking feature of the results was the much greater killing power of "active chlorine" and permanganate in comparison with that of all the other disinfectants tested. This seems to indicate that phage is most susceptible to oxidation, although the relatively low efficiency of peroxide is anomalous from this point of view. It is interesting to note that Clifton (5) found that inactivation of phage by methylene blue was an oxidation process.

In the case of alcohol it was not of course possible to mix equal parts of the watery phage preparation with the alchol. Thus there was present in the reaction mixture only 10–30 % of phage. This slight discrepancy between

alcohol and the other disinfectants was not considered important in view of the very delicate test used for detection of the presence of surviving phage. Experiments with some of the other disinfectants showed that within limits the actual concentration of a powerful phage in the reaction mixture was not of major importance in determining the lethal concentration of disinfectant. Concentrations of alcohol above 85% showed a tendency to be less lethal than concentrations between 80 and 85%, possibly because water was necessary for the phage-inactivating reaction to proceed.

It was evident from the results that, apart from considerations of suitability under dairying conditions, active chlorine, permanganate, hydrogen peroxide, and formaldehyde were the only disinfectants with sufficient power to merit consideration. Of these active chlorine was on all counts the most suitable.

The experimental results given in Table 1 were obtained with one phage preparation. It was obviously necessary to determine whether all our phages for lactic streptococci reacted similarly. Actually it was found that eight phage races, all prepared in the same batch of milk, needed varying concentrations of active chlorine for complete inactivation of phage within 1 min. The lethal concentrations and the protein and lactose contents and *p*H values of the phages are given in Table 2.

Table 2. *Composition of phage preparations in relation to inactivation by means of active chlorine*

Minimum lethal concentration of active chlorine: exposure 1 min. %	Phage race	Protein %	Lactose %	*p*H
0·05	*hp*	0·49	4·53	5·99
	rw	0·49	4·42	6·11
	l	0·51	4·47	5·97
	r	0·60	4·42	6·05
	ud_5	0·61	4·56	6·11
0·06	*b*	0·71	4·37	5·73
	k	0·64	4·33	6·01
Over 0·06	*j*	0·84	4·27	5·46

It was evident that the most likely cause for the slight differences in resistance of the phage races was the varying protein content (correlated to some extent with differences in lactose content and *p*H value) of the preparations. This prediction was confirmed by adjusting all the preparations to a common protein content of 0·49% when it was found that all the phage races were destroyed by exposure to 0·05% active chlorine within 1 min.

The other disinfectants were not tested in the different phage races since it seemed clear that active chlorine was the most suitable disinfectant for the practical purpose we had in mind. It seems likely, however, that there would not be marked differences between phages for the lactic streptococci in their reaction to the other disinfectants.

Discussion

The work which has been described was designed to give indications of the most promising means for inactivating phage on utensils and in the surroundings of dairy factories. Apparently active chlorine is by far the most effective and most convenient agent for the destruction of phage. Trials under commercial conditions are necessary to show whether the promise of the laboratory experiments are borne out in practice or whether some modifying factor has been overlooked. It has recently been found that air-borne contamination with phage is of importance under some circumstances and it is proposed to attempt to overcome this by a spray treatment with solution containing active chlorine. It has proved impossible so far to reproduce in the laboratory air-borne infection as it occurs under commercial conditions. The effectiveness or otherwise of active chlorine in this connexion thus remains to be determined.

Summary

The times needed for the complete inactivation of bacteriophages for the lactic streptococci by several chemical disinfectants in various strengths were determined. The phage preparations, consisting essentially of whey, contained the usual amounts of protein and lactose, so that the results obtained must be considered applicable only under the defined conditions. Hydrogen and hydroxyl ions were found to inactivate phage when they were present in sufficient concentration but their effects between *p*H values of 4 and 7 were negligible over several days at room temperature.

By far the most effective of the disinfectants were active chlorine and permanganate, which suggests that the inactivation of phage is most readily brought about by oxidation.

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252. THE EFFECT OF COMMERCIAL PASTEURIZATION AND STERILIZATION ON THE VITAMIN B_1 AND RIBOFLAVIN CONTENT OF MILK AS MEASURED BY CHEMICAL METHODS

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Our earlier biological tests showed that commercial pasteurization causes a definite though small loss in the vitamin B complex of milk (1) and that commercial sterilization destroys some 30% of vitamin B_1 but has no apparent adverse effect on vitamin B_2 which in this test was probably synonymous with riboflavin (2).

We have now applied chemical tests for vitamin B_1 and riboflavin to commercially pasteurized and commercially sterilized milks obtained in the same way and from the same sources as before. Vitamin B_1 was at first measured by the original method of Jansen (3). Later, when it was found that phosphorylated and protein-bound vitamin B_1 do not react in this technique, the total aneurin of milk was measured after incubation with takaphosphatase and with pepsin (4, 5, 6).

Riboflavin was measured by the method of Whitnah *et al.* (7) and later by that of Emmerie (8). When the former was used it was often noticed that the apparent riboflavin content of pasteurized milk was higher than that of the corresponding raw milk. This was probably due to the presence in milk of riboflavin-protein complexes which break down on heating (8) but are little affected by treatment with trichloroacetic acid in the cold.

Experimental

(1) *Pasteurized milk*

(*a*) *Vitamin* B_1. In Table 1 the results of three series of tests are given. In two of them only the "free" vitamin B_1 was measured. In the last the effect of pasteurization on the total aneurin of milk was also studied.

The first experiment showed a loss of over 20% of the "free" vitamin B_1; in the other two the loss was smaller, some 10%. Moreover, in the last experiment the loss in total vitamin B_1, which is a more reliable indication of biological effect, was also only about 10%. On the whole the chemical test agrees well with the results of previous biological measurements (1) which indicated that the destruction of vitamin B_1 when milk was pasteurized was not serious.

Table 1. *Effect of commercial pasteurization on the vitamin* B_1 *of milk*

		Vitamin B_1 i.u./100 ml.		
Exp. no.	Date	Raw milk	Pasteurized milk	% destruction
1*	7. ii. 38	10·3	8·2	20·4
	8. ii. 38	11·2	8·6	23·2
	10. ii. 38	9·8	6·9	29·5
	11. ii. 38	9·6	7·4	22·9
	15. ii. 38	10·6	8·4	20·8
	Mean	10·3	7·9	23·4
2*	14. vi. 38	6·4	5·9	7·8
	15. vi. 38	8·3	6·1	26·5
	16. vi. 38	6·8	6·2	8·8
	29. vi. 38	7·6	6·8	10·5
	30. vi. 38	7·3	6·5	11·0
	Mean	7·3	6·3	13·6
3*	25. vi. 39	7·4	6·1	17·6
	26. vi. 39	4·9	4·4	10·2
	27. vi. 39	6·2	5·9	4·8
	28. vi. 39	5·7	5·3	7·0
	Mean	6·0	5·4	10·0
3†	25. vii. 39	11·6	10·8	6·9
	26. vii. 39	11·4	10·5	7·9
	27. vii. 39	12·5	10·6	15·2
	28. vii. 39	10·0	9·3	7·0
	Mean	11·4	10·3	9·6

* "Free" vitamin B_1 measured. † Total vitamin B_1 measured.

(*b*) *Riboflavin.* The figures for three series of tests for riboflavin are given in Table 2. The first, in which the unmodified method of Whitnah *et al.* (7) was used, shows the apparent increase of riboflavin after pasteurization. In the second the raw milk samples were analysed as such and also after keeping in boiling water for 10 min.

In the third the method of Emmerie (8) was used which involves heating in methanol solution, but measurements were also carried out in which the heating was omitted.

Table 2. *Effect of commercial pasteurization on the riboflavin of milk*

		Riboflavin μg./100 ml.			
		Raw milk		Pasteurized milk	
Exp. no.	Date	Untreated	Heated	Untreated	Heated
1	7. ii. 38	80	—	88	—
	8. ii. 38	86	—	102	—
	10. ii. 38	123	—	140	—
	11. ii. 38	92	—	110	—
	15. ii. 38	100	—	108	—
	Mean	96	—	110	—
2	14. vi. 38	121	134	136	—
	15. vi. 38	148	157	152	—
	16. vi. 38	145	155	156	—
	29. vi. 38	143	161	160	—
	30. vi. 38	150	160	158	—
	Mean	141	153	152	—
3	25. vii. 39	174	179	174	174
	26. vii. 39	119	137	129	138
	27. vii. 39	148	154	153	163
	28. vii. 39	157	174	184	196
	Mean	149	161	160	168

The figures show conclusively that the riboflavin of milk is not adversely affected by pasteurization.

(2) *Sterilized milk*

The tests on sterilized milk were carried out under similar conditions and at approximately the same times as those on the pasteurized milk.

(*a*) *Vitamin* B_1. The results will be found in Table 3.

Table 3. *Effect of commercial sterilization on the vitamin* B_1 *of milk*

		Vitamin B_1 i.u./100 ml.		
Exp. no.	Date	Raw milk	Sterilized milk	% destruction
1*	17. ii. 38	9·2	4·8	47·8
	18. ii. 38	12·8	7·9	38·3
	24. ii. 38	7·1	3·8	46·5
	25. ii. 38	3·9	2·1	46·1
	1. iii. 38	6·1	3·3	45·9
	Mean	7·8	4·4	44·9
2*	15. vi. 38	6·1	5·1	16·4
	16. vi. 38	6·9	5·0	27·9
	17. vi. 38	6·5	5·3	18·5
	18. vi. 38	8·3	5·2	37·3
	Mean	6·9	5·1	26·1
3*	6. vii. 39	6·8	4·7	30·9
	13. vii. 39	7·0	3·5	50·0
	20. vii. 39	5·2	3·4	34·6
	27. vii. 39	6·8	5·0	26·5
	10. viii. 39	6·3	3·7	41·3
	17. viii. 39	7·9	4·3	45·6
	Mean	6·7	4·1	38·8
3†	6. vii. 39	10·5	4·9	53·3
	13. vii. 39	12·6	6·4	49·2
	20. vii. 39	10·3	4·7	54·4
	27. vii. 39	11·8	5·0	57·6
	10. viii. 39	9·2	5·0	45·6
	17. viii. 39	12·6	5·5	56·3
	Mean	11·2	5·3	52·7

* "Free" vitamin B_1 measured. † Total vitamin B_1 measured.

There was a marked loss of vitamin B_1 in all three series of tests, the destruction of the "free" vitamin varying from 26 to 45 %. The loss of total vitamin B_1 in the last experiment was as high as 53 %. In biological tests on comparatively small groups of rats Henry & Kon (2) obtained values of from 17 to 46 % loss. The agreement is reasonable and the results obtained support the earlier findings. A consideration of the figures for the third experiment in Table 3 shows that the combined form of vitamin B_1 is more readily destroyed by sterilization than the "free" form. Values for combined vitamin B_1 can be obtained by subtracting figures for "free" from those for total vitamin B_1 and calculation shows that the destruction of this form amounts to 73 % or almost double that of the free vitamin B_1.

(*b*) *Riboflavin.* The results are summarized in Table 4. They show that riboflavin is sufficiently heat-stable to withstand the drastic treatment of sterilization of milk and confirm in this respect the earlier findings (2) based on biological tests.

Table 4. *Effect of commercial sterilization on the riboflavin content of milk*

		Riboflavin μg./100 ml.			
		Raw milk		Sterilized milk	
Exp. no.	Date	Untreated	Heated	Untreated	Heated
1	17. ii. 38	102	108	106	—
	18. ii. 38	92	96	110	—
	19. ii. 38	103	105	101	—
	24. ii. 38	106	115	127	—
	25. ii. 38	93	86	93	—
	1. iii. 38	87	103	103	—
	Mean	97	102	107	—
2	15. vi. 38	142	158	152	—
	16. vi. 38	139	160	143	—
	17. vi. 38	147	156	162	—
	18. vi. 38	132	144	140	—
	Mean	140	154	149	—
3	6. vii. 39	172	189	179	187
	13. vii. 39	125	139	133	144
	20. vii. 39	167	196	185	194
	27. vii. 39	166	180	170	176
	10. viii. 39	155	169	155	162
	17. viii. 39	169	180	172	174
	Mean	159	175	166	173

The data in Tables 2 and 4 show incidentally that milk is definitely richer in riboflavin in summer than in winter. The seasonal fluctuation in this factor will be reported in a separate paper.

Summary

1. Fluorimetric tests applied to commercially pasteurized and commercially sterilized milk showed that in the former some 10% and in the latter up to 50% of vitamin B_1 was destroyed in the course of the heat treatment.

2. Riboflavin withstood both treatments without loss.

3. These findings are in satisfactory agreement with the results of earlier biological tests.

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253. THE MEASUREMENT AND SIGNIFICANCE OF *p*H VALUES IN CHEESEMAKING

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Introduction

Acidity may be regarded as the keystone in the practice of cheesemaking as, although the other major factors (i.e. temperature and size of cut) can be readily measured, acidity is a factor that is somewhat more difficult to measure and control. Before the introduction of the acidimeter by F. J. Lloyd (1), cheesemakers had no conception of acidity as such, although for centuries they had made use unconsciously of several properties which in reality afforded an indirect measure of acidity. Methods for measuring acidity in cheesemaking may be classified as:

(1) Indirect and empirical: (*a*) "lactic acid" aroma, (*b*) rennet test, (*c*) "feel" of the curd, (*d*) hot iron test.

(2) Titration of the whey with alkali: titratable acidity.

(3) *p*H determination (whey or curd): A, colorimetric: (*a*) Wulff tester, (*b*) dilution method; B, potentiometric: (*a*) quinhydrone electrode, (*b*) glass electrode.

The most suitable accurate method for determining *p*H values in cheesemaking is the quinhydrone electrode. But apart from the inherent disadvantages of complexity and expense, the humidity of cheese factories and dairies introduces serious technical drawbacks to the use of a potentiometer. An important advantage of the quinhydrone electrode is that the *p*H of the *curd* can be directly determined (2). Differences between the *p*H values of the curd and the whey in which it is floating may be as high as 0·2 or even 0·3 unit. Errors such as this may be minimized by squeezing out whey from the curd and using the last few drops for the determination of *p*H. Summing up, it may be stated that while the quinhydrone electrode is feasible for large scale factory work where a laboratory and trained chemist are available, it is not practicable in smaller factories and farms. It follows, therefore, that some form of colorimetric method is the only practicable method of sufficient accuracy for control work.

The uncertainty of the *p*H-titratable acidity relationship, and the fact that it is *p*H rather than titratable acidity that controls curd behaviour, makes a simple method of *p*H measurement the most urgent need of the cheesemaker. Colorimetric methods, involving only inexpensive apparatus and a simple technique, are therefore of great importance. The turbidity of milk and whey

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prevents the use of comparator methods in the ordinary way and special devices must be employed, such as absorption of the material into an indicator-impregnated cellophane slip, as in the Wulff tester (3), or dilution of the turbid milk or whey before using a comparator, as described by Sharp & McInerney (4). The dilution effect on the *p*H values of butter serum has also been investigated by Hunziker *et al.* (5), and McDowall & McDowell (6) have used the colorimetric dilution method for cream plasma. The present work is concerned with the application of this method to cheesemaking.

The Wulff tester has been investigated by Aschaffenburg (3) who has shown that an accuracy of about $\pm 0{\cdot}1$ *p*H unit can be obtained. One of us (J. G. D.) has tried the Wulff tester and obtained results similar to those of Aschaffenburg, but its inherent disadvantages of time required for a test (about 2 min.) and cost (each test costs about 1*d.*), limit its application in cheesemaking.

Experimental results

The colorimetric dilution method

This method was adopted as the most practicable method of sufficient accuracy to justify its use in cheesemaking. Milk or whey is diluted 20 times, indicator is added and the *p*H estimated in a comparator such as the Lovibond apparatus (4). We have studied this method in cheesemaking practice and found a mean error of about 0·05, the quinhydrone electrode being taken as a standard. It is rapid, only 15 sec. being required for a determination, and cheap (five tests costing about 1*d.*) and so forms a practicable method of *p*H testing for the cheesemaker. Corrections for dilution are obtained from a table (Table 1).

In comparing this technique with that suggested by Sharp & McInerney (4) the following conclusions were reached:

(1) The correction values for diluted whey were found to be identical with those given by these workers (4) (see Tables 1 and 2).

(2) Bromcresol purple is, as far as the *p*H range is concerned, suitable for testing wheys. The range is 5·2–6·8 which is roughly that of diluted (1 : 20) wheys in ordinary Cheddar practice. We were, however, not satisfied on many occasions with the matching, the experimental tube of whey + indicator being too blue (i.e. having insufficient red). Chlorphenol red gave better matching than bromcresol purple but this indicator is rather variable so that its use is to be avoided. Other indicators were therefore investigated. Satisfactory matching was obtained with the B.D.H. 4460 indicator, which is a screened methyl red. We therefore recommend this indicator for wheys which have *p*H values of less than 5·8 (see Tables 1, 2 and 4).

(3) Phenol red did not match satisfactorily in diluted milk and was affected by the fat content (see Table 3). This indicator was selected by Sharp & McInerney (4) for estimating the *p*H of milk. A further disadvantage is that in any case the *p*H of diluted milk (*c.* 7·1) lies in the extreme acid range of

phenol red. Exploration of other indicators resulted in the choice of bromthymol blue, which has the advantages of matching perfectly, and because of the different corrections for milk and whey, can also be used for whey of *p*H greater than 5·8 (Table 4). It is necessary, however, to point out that bromthymol blue behaves anomalously in milk, a fact recognized by Sharp & McInerney (4) who rejected this indicator. The correction is relatively large, the apparent *p*H being in fact about 0·2 unit *less* than that of the *undiluted* milk. The indicator is unaffected by fat (see Table 3), the anomalous behaviour in milk being due presumably to casein. Nevertheless, we feel that the advantages of perfect matching, absence of sensitivity to fat, and the simplicity resulting from the use of one indicator for milk and whey, more than outweigh the disadvantage of anomalous behaviour. Ease of working and ability to distinguish *changes* in *p*H are, in our opinion, of prime importance in this problem.

Table 1. *Corrected values*

Indicator	Reading	Actual *p*H	
		Milk	Whey
Phenol red	7·4	6·86	—
	7·3	6·76	—
	7·2	6·66	6·94
	7·1	6·56	6·84
	7·0	6·46	6·74
	6·9	6·36	6·64
	6·8	6·26	6·54
	6·7	6·16	6·44
Bromcresol purple	6·6	6·06	6·34
	6·5	5·96	6·24
	6·4	5·86	6·15
	6·3	5·76	6·05
Chlorphenol red	6·2	5·66	5·96
	6·1	5·57	5·87
	6·0	5·48	5·77
	5·9	5·40	5·68
	5·8	5·32	5·58
	5·7	5·24	5·48
	5·6	5·17	5·38
	5·5	5·11	5·28
	5·4	5·05	5·18
	5·3	4·99	5·08
	5·2	4·92	4·98
	5·1	4·86	4·88
	5·0	4·78	4·78
Bromthymol blue	6·0–6·8	Add +0·22	Subtract 0·2
4460 indicator	5·2–6·0	—	Subtract 0·2

The values, except those for bromthymol blue and 4460, are from Sharp & McInerney (4).

Table 2. *Mean correction values for wheys*

*p*H range	Scotch Cheddar		Dunlop	Cheshire	Derby	All varieties	Sharp and McInerney's values
	Raw	Pasteurized					
6·5 and above	−0·20	−0·26	−0·25	−0·26	−0·30	−0·26	−0·26
6·0–6·4	−0·22	−0·25	−0·25	−0·26	−0·29	−0·25	−0·24
5·9 and below	−0·20	—	−0·23	−0·19	—	−0·20	−0·22

The indicators used were bromcresol purple and 4460.

Table 3. *Determination of pH of milks with phenol red and bromthymol blue*

*p*H of original milk	Apparent *p*H		Correction for	
	Phenol red	Bromthymol blue	Phenol red	bromthymol blue
6·68	7·15	6·47	−0·47	+0·21
6·66	7·10	6·40	−0·44	+0·26
6·62	7·10	6·35	−0·48	+0·27
6·62	7·10	6·40	−0·48	+0·22
6·58	7·10	6·35	−0·52	+0·23
6·58	7·05	6·40	−0·47	+0·18
6·55	7·00	6·40	−0·45	+0·15
6·53	7·05	6·33	−0·52	+0·20
6·44	7·00	6·27	−0·56	+0·17
6·41	6·90	6·15	−0·49	+0·26
6·35	6·80	6·05	—	+0·30
		Mean values	−0·49	+0·22

Fat error of indicators

Cream line % after centrifuging	*p*H of original milk	Apparent *p*H			
		Phenol red		Bromthymol blue	
		Whole	Centrifuged	Whole	Centrifuged
10·0	6·57	7·10	7·05	6·27	6·25
9·8	6·64	7·07	7·00	6·37	6·40
7·7	6·51	7·00	6·90	6·30	6·30
5·5	6·75	7·27	7·10	6·47	6·50
5·0	6·58	7·10	6·95	6·33	6·36
4·7	6·56	7·07	7·00	6·37	6·35

Phenol red invariably matched poorly, appearing too brown (i.e. insufficient red in experimental tube). Bromthymol blue always matched excellently.

Table 4. *Typical examples in the determination of pH of wheys*

*p*H of original whey (quinhydrone)	Apparent *p*H (i.e. *p*H of diluted whey)	Difference	Variety
	(i) Bromthymol blue		
6·37	6·53	−0·16	Derby
6·43	6·50	−0·07	Cheshire
6·26	6·45	−0·19	Derby
6·21	6·35	−0·14	Dunlop
6·14	6·35	−0·21	Scotch Cheddar
6·09	6·35	−0·26	Cheshire
6·16	6·33	−0·17	Derby
6·25	6·30	−0·05	Cheshire
6·02	6·25	−0·23	Dunlop
5·99	6·25	−0·26	Scotch Cheddar
5·94	6·23	−0·29	Cheshire
	(ii) B.D.H. 4460 indicator		
5·68	5·93	−0·25	Dunlop
5·76	5·90	−0·14	Cheddar
5·71	5·90	−0·19	Derby
5·67	5·90	−0·23	Dunlop
5·73	5·85	−0·22	Cheddar
5·52	5·70	−0·18	Cheshire
5·42	5·60	−0·18	Cheshire
5·18	5·45	−0·27	Cheshire

Special disks graduated in 0·1 unit which permit rapid estimation to 0·05 % of a *p*H unit have been prepared for us by the Tintometer Ltd. in conjunction with British Drug Houses. The ranges and correction values are given in Table 1.

Apparatus required

A Lovibond comparator.

Special disks for bromthymol blue and 4460 indicator calibrated in 0·1 *p*H unit. Alternatively, standard disks for these indicators or for bromcresol purple or chlorphenol red may be used.

Tubes calibrated at 10 ml.

Rubber-teated pipettes measuring 0·5 ml. and 1 ml.

Boiled distilled or rain water in a CO_2-protected aspirator bottle.

Method

Testing the distilled water

Fill up a tube to the 10 ml. mark and add 0·5 ml. of each indicator solution in turn. If the *p*H is not $<5{\cdot}6$ or $>7{\cdot}0$ the water is satisfactory. The chief sources of error are (1) absorption of CO_2 leading to too acid values, and (2) presence of free alkali in the glass leading to too alkaline values. Ordinary soda glass must, therefore, not be used. On no account should an ordinary wash bottle be used for adding the distilled water, as blowing into such a flask may result in values 0·3 *p*H unit too low. The use of freshly prepared but not boiled distilled water gave readings about 0·05 too low.

Testing milk and whey

Add 0·5 ml. of milk or whey to a tube, make up to 10 ml. with boiled distilled water, add 0·5 ml. of the appropriate indicator and match the colour against the disk. Estimate to 0·05 *p*H unit. With practice estimations to 0·03 (i.e. dividing the colour step mentally into thirds) can be made. For corrected readings see Table 1.

Discussion

Titratable acidity, pH values and buffer values of milk and whey

The introduction of the acidimeter into Cheddar cheesemaking by Lloyd may be regarded as the first attempt to put Cheddar cheesemaking on a scientific basis, although at that time the conception of *p*H was unknown. Since, normally, there is a fairly constant relationship between *p*H and titratable acidity of milk or whey, a given titratable acidity approximately defines the *p*H. The only weakness of working with titratable acidities is that variation in buffer value which may arise from differences in breed, method of feeding, mastitis, variation in the acid-base equilibrium between whey and curd, etc. will upset this relationship, so that under such circumstances titratable acidities will not be a reliable guide to the *p*H. An illustration of

this is the observation, long known to expert cheesemakers, that in a milk of high initial titratable acidity a greater increase of acidity (e.g. 0·03 as against 0·02) must be obtained in order to arrive at the right point for rennetting.

*The significance of p*H *measurement in cheesemaking*

It is not the purpose of this paper to suggest that cheesemakers should immediately adopt *p*H control of manufacture instead of using titratable acidities, as much work requires to be done before this method can be finally recommended. There is little doubt, however, that cheesemaking will ultimately be established on a *p*H basis (cf. refs. (7-13)).

The chief interest at present lies in comparing *p*H values and titratable acidity measurements. Each of these may be regarded as a characteristic, incomplete in itself, but in conjunction with the other affording a most useful check on the behaviour of the cheese. We have already instanced the fact that experienced cheesemakers compensate for the increased buffer value of milk of high initial acidity. A further point of interest is the observation, well known to experts, that titratable acidity and the "hot iron" test begin to diverge in August. When this occurs cheesemakers usually rely on the "hot iron" test.

Buffer values of whey

As phenolphthalein turning at *p*H 8·4 is generally used in dairy practice the buffer value has been calculated according to the formula:

$$\text{B.V.} = \frac{(\text{Ml. } N/9 \text{ NaOH per 10 ml. milk or whey}) \times 11{\cdot}1}{8{\cdot}4 - p\text{H}}$$

$$= \frac{\text{titratable acidity} \times 111}{8{\cdot}4 - p\text{H}}.$$

The buffer value of whey depends largely on the content in phosphate, lactic and citric acids (14), and soluble protein (chiefly albumin and decomposition products of casein as the result of rennet action). It follows, therefore, that the buffer value of whey affords a most valuable simple method of determining if the manufacture is proceeding normally. The buffer value rises continuously during making and figures illustrating this for four varieties of cheese are given in Table 5.

The abrupt rise in *p*H after salting, with only a slight or even with no corresponding fall in titratable acidity (cf. (15)), and the effect of pasteurization of milk on the buffer value of whey are of particular interest. The former is presumably due to the solution of buffering brine-soluble casein decomposition products in the salty whey, and the latter to the partial precipitation of calcium phosphate and heat coagulable proteins. For any stage in the making process, the most important factor controlling the buffer value of the whey is the rate of working. Pasteurization results in slightly lower buffer values although this effect may be masked by differences in the rate of working.

Table 5. *Typical pH and buffer values during cheesemaking*

Stage	Scotch Cheddar		Dunlop		Cheshire		Derby	
	*p*H	B.V.	*p*H	B.V.	*p*H	B.V.	*p*H	B.V.
(1) Milk at rennetting	6·51		6·51		6·52		6·54	
	6·56 P		6·56		6·58		6·56	
	6·52							
(2) Whey at cutting	6·52	*7·7*	6·56	*7·8*	6·52	*7·8*	6·56	*7·5*
	6·52 P	*7·1*	6·51	*7·7*	6·56	*7·8*		
	6·47	*7·8*						
(3) Max. scald	6·14		6·21	*7·4*	6·43	*7·9*	6·37	*7·4*
	6·30	*8·2*	6·38	*8·0*	6·38	*8·0*	6·40	—
	6·25 P	*7·5*	6·26	*7·5*	6·43	*7·9*		
	6·21	*7·9*			6·33	*7·8*		
	6·16	*7·9*						
(4) Settling curd	5·99	*9·0*	6·02	*8·9*	6·09	*8·2*	6·26	*7·5*
	6·07	*8·6*	5·95	*8·8*	6·20	*8·3*	6·35	*7·6*
	6·09 P	*8·3*	5·85	*8·9*	6·25	*8·3*		
	5·78	*10·2*			6·25	*8·5*		
	5·88	*9·5*						
(5) Running whey	5·80	*9·8*	5·73	*11·2*	5·95	*9·1*	6·16	*8·4*
	5·69	*11·3*			5·94	*9·5*		
	5·76	*10·5*	5·67	*11·0*	6·07	*8·3*	6·09	*7·9*
	5·63	*12·1*	5·78	*10·0*	5·88	*10·1*		
	5·78 P	*11·0*						
(6) Piling	5·73	*10·6*	5·67	*11·0*	5·52	*13·5*	5·68	—
					5·63	*13·4*		
					5·42	*16·1*		
(7) Milling							5·28	*19·6*
(8) After salting					5·72	*24·6*	5·82	*23·2*
(9) From press					5·71	*26·6*		

B.V. = buffer value.
P = cheese made from pasteurized milk.
Values for corresponding raw-milk cheese bracketed.

Since titratable acidities, *p*H values and buffer values are interrelated, any one being determinable from the other two, it is not necessary to plot all three values. For the establishment of a norm for any variety of cheese and for the investigation of the effects of season, type of milk and of other factors on the making process, the most convenient way is to plot times on the horizontal axis against *p*H values and titratable acidities on the vertical axis. The extent of divergence of the two curves so obtained is a measure of the buffer value of the whey, and thus calculation is avoided. At the same time stages in the making process can be related to time, *p*H and titratable acidity values. In this way the behaviour of a vat can be epitomized in one diagram and any abnormality is readily recognized.

With slow-working curds, the titratable acidity may increase without any appreciable change in *p*H, presumably because the rate of entry of buffering substances into the whey relative to that of lactic acid formation is higher than the normal.

SUMMARY

1. The application of the colorimetric dilution method for the determination of *p*H values in cheesemaking is discussed and the necessary precautions for accuracy described.

2. Bromthymol blue is recommended for milk and whey of *p*H > 5·8 and the B.D.H. 4460 indicator for whey of *p*H < 5·8.

3. Titratable acidities and *p*H values each afford an incomplete picture of the working of a curd. Together they afford a valuable indication of the normality or otherwise of the making process.

4. The buffer value of whey rises steadily during manufacture. The *p*H rises abruptly after salting, although the titratable acidity continues to increase.

5. The buffer value of whey is controlled chiefly by the rate of working but tends to be lower with pasteurized milk.

6. A diagrammatic method for summarizing the "behaviour of a vat" and for detecting abnormal working is described.

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254. THE DETERMINATION OF *p*H IN MILK AND WHEY BY MEANS OF COLOUR INDICATOR PAPER

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On account of a paper published in this *Journal* by R. Aschaffenburg(1) concerning the colorimetric determination of *p*H in milk and whey by means of the "Wulff" *p*H tester, the results recently obtained in this laboratory by using Lyphan colour indicator paper will be briefly communicated.

The experiments were carried out with the milk and whey of normal cows and also with the milk and whey of cows known to be infected with streptococcal mastitis. Furthermore, normal milks were soured by storing during a few days and one sample was artificially alkalinized by adding sodium hydroxide. The different samples of whey prepared from normal milks were artificially soured and made alkaline by adding lactic acid or sodium hydroxide. The colour indicator paper was thus tested over the total range of *p*H encountered in normal and pathological conditions. The experimental results obtained were checked by potentiometric *p*H determinations by means of the glass electrode. In experiments carried out over many years the latter method has been shown to furnish the best results in the determination of *p*H of different fluids of biological origin, including milk(2).

Procedure with colour indicator paper[1]

In preparing lyphan paper strips of filter paper are impregnated with a colour scale of five to eight colours showing *p*H intervals of 0·2 or 0·3 *p*H unit, covering a definite *p*H range. In the middle there is a space impregnated with "neutral" indicator which, on immersing the strip, assumes a colour in accordance with the *p*H of the fluid to be tested. This colour may be compared with the colour scale on the strip. As a rule comparison is not hindered by turbidity or by the specific colour of the fluid, because the colours of the scale as well as the colour of the "unknown" are changed by those factors in the same way.

Lyphan paper is immersed in the fluid to be tested only *for a moment* and is immediately exposed to the air. Colour comparison must be made in clear diffuse daylight or in electric light *within* 1 *to* 2 *sec. after exposure to the air.*

Whey was prepared by adding 10 ml. of 0·5 % rennet[2] solution to 500 ml. of milk at 35° C., gradually raising the temperature to 45° C. within ½ hr., keeping the temperature at 45° for 15 min. and filtering through paper.

[1] Lyphan paper, Dr Gerh. Kloz, 80a Delitzscherstr., Leipzig, N. 21.

[2] The concentration of the dry preparation of rennet used was 1 : 100,000.

Table 1. *Milk of normal cows (individual cows)*[1]

No.	Titratable acidity (ml. of ¼ normal alkali per 100 ml. of milk)	*p*H: ° C.	*p*H: Glass electrode	*p*H: Lyphan M 25	Difference	
1*a*	7·5	21	6·65	6·6	−0·05	
b	7·8	20	6·59	6·6	+0·01	
c	16·7	20·6	5·69	5·7	+0·01	L 665: *p*H about 5·4 (comparison very bad)
2*a*	7·4	21	6·61	6·6	−0·01	
b	8·5	20	6·44	6·5	+0·06	Little difference in colour
c	35·0	20·6	4·80	4·8 (L 662)	0·00	
3*a*	7·9	21	6·60	6·6	0·00	
b	8·9	20	6·43	6·45	+0·02	
c	33·4	20·8	4·43	4·4 (L 662)	−0·03	
4*a*	7·0	21	6·64	6·6	−0·04	
b	8·9	20	6·29	6·15	−0·14	
				6·2	−0·09	
c	33·1	20·8	4·41	4·4 (L 662)	−0·01	
5*a*	9·3	21	6·52	6·6	+0·08	Little difference in colour
b	14·1	20	5·87	5·75	−0·12	L 665: *p*H = 5·6, little difference in colour
c	37·5	20·4	4·41	4·3 (L 662)	−0·11	
6*a*	7·4	21	6·60	6·6	0·00	
b	8·8	20	6·29	6·3	+0·01	
c	33·6	20·8	4·40	4·3 (L 662)	−0·10	
7*a*	7·1	20·5	6·66	6·6	−0·06	
b	9·2	20	6·30	6·3	0·00	
c	34·4	20·6	4·42	4·4 (L 662)	−0·02	
8*a*	7·7	20·5	6·61	6·6	−0·01	
b	10·8	20	6·12	6·0	−0·12	
c	35·3	20·6	4·34	4·3 (L 662)	−0·04	
9*a*	7·4	20·5	6·63	6·6	−0·03	
b	9·0	20	6·41	6·45	+0·04	
c	39·1	20·6	4·47	4·4 (L 662)	−0·07	
10*a*	7·3	20·5	6·60	6·6	0·00	
b	8·7	20	6·39	6·45	+0·06	
c	35·6	20·6	4·41	4·4 (L 662)	−0·01	
11*a*	8·5	20·5	6·55	6·6	+0·05	
b	9·8	20	6·36	6·45	+0·09	
c	34·2	20·6	4·60	4·5 (L 662)	−0·10	
12*a*	6·2	20·5	6·70	6·6	−0·10	L 669: *p*H = 6·6; L 665: no comparison possible
b	13·2	20	5·70	5·5	−0·20	L 662: *p*H slightly above 5·4
c	33·2	20·6	4·34	4·3 (L 662)	−0·04	
13*a*	7·4	20	6·72	6·6	*c.* −0·12	L 669: *p*H lower than 6·6
b	6·4	20	6·77	6·6	*c.* −0·17	L 669: *p*H slightly lower than 6·6
c	5·4	20	6·92	<6·6 (L 669)	*c.* −0·4	
d	4·4	20	7·09	*c.* 6·6 (L 669)	*c.* −0·5	
e	3·4	20	7·27	6·6 (L 669)	−0·67	
f	2·4	20	7·50	6·7 (L 669)	−0·80	L 671: *p*H much lower than 7·5
g	1·4	20	7·78	6·9 (L 669)	−0·88	L 671: *p*H slightly lower than 7·5
h	0·4	20	8·09	7·1 (L 669)	−0·99	
				7·6 (L 671)	−0·49	
i	−0·6	20	8·44	7·2 (L 669)	−1·24	
				7·9 (L 671)	−0·54	
j	−1·6	20	8·87	7·8 (L 669)	−1·07	
				c. 8·3 (L 671)	*c.* −0·57	Colour with L 671 changes rapidly. L 674: *p*H may be slightly lower than 8·2
				8·2 (L 674)	*c.* −0·67	

Nos. 1–12: stored at 22° C.: *a*, about 4 hr.; *b*, 28 hr.; *c*, 52 hr.

Nos. 13*b*–13*j* prepared from the normal milk sample No. 13*a* in adding to 100 cm.³ of milk, respectively 0·25, 0·50, 0·75, 1·00, 1·25, 1·50, 1·75, 2·00 and 2·25 cm.³ of 1 normal sodium hydroxide: *p*H determined after 2 hr. storing at room temperature.

[1] Another series of thirty milks (see text).

Table 2. *Whey from normal cow's milk (individual cows)*

No.	ml. of 1 *N* lactic acid or NaOH added to 30 ml. of whey	*p*H (21° C.) Glass electrode	Lyphan	Difference	
1*a*	—	6·59	6·6 (M 25)	+0·01	
			6·4 (L 665)	−0·19	
			<6·6 (L 669)	—	
b	0·10	6·31	6·3 (M 25)	−0·01	L 665: no comparison possible
c	0·25	5·64	5·6 (M 25)	−0·04	
			5·5 (L 665)	—	
d	0·50	4·94	4·6 (L 662)	−0·34	
e	0·20	7·36	6·9 (L 669)	−0·46	
f	0·40	8·36	7·8 (L 671)	−0·56	
g	0·50	9·14	8·2 (L 671)	−0·94	
			8·2 (L 674)	−0·94	
2*a*	—	6·80	>6·6 (M 25)	*c.* −0·20	
			6·4 (L 665)	−0·40	
			<6·6 (L 669)	*c.* −0·20	
b	0·10	6·38	6·3 (M 25)	−0·08	
			c. 6·1 (L 665)	—	
c	0·25	5·67	5·5 (M 25)	−0·17	
			5·5 (L 665)	−0·17	
d	0·50	4·94	4·6 (L 662)	−0·34	
e	0·20	7·72	<7·5 (L 671)	*c.* −0·22	
			7·0 (L 669)	−0·72	
f	0·40	9·37	8·3 (L 674)	−1·07	
			8·4 (L 671)	−0·97	
g	0·50	9·88	8·8 (L 674)	−1·08	
			>8·7 (L 671)	—	
3*a*	—	6·48	6·45 (M 25)	−0·03	L 665: about 6·4
b	0·10	6·09	6·2 (M 25)	+0·11	
			6·1 (L 665)	+0·01	
c	0·25	5·38	5·5 (L 665)	+0·12	
d	0·50	4·63	4·50 (L 662)	−0·13	
e	0·10	6·87	>6·6 (M 25)	—	L 665: 6·7; L 669: about 6·6
f	0·20	7·27	6·9 (L 669)	−0·37	
g	0·30	7·82	7·2 (L 669)	—	
4*a*	—	6·55	6·45 (M 25)	−0·10	L 665: about 6·4
b	0·10	6·10	6·15 (M 25)	+0·05	
			6·1 (L 665)	0·00	
c	0·25	5·44	5·5 (L 665)	+0·06	
d	0·50	4·78	4·7 (L 662)	−0·08	
e	0·10	6·95	>6·6 (M 25)	*c.* −0·3	L 665: >6·7; L 669: about 6·6
f	0·20	7·31	6·9 (L 669)	−0·41	
g	0·30	7·82	7·1 (L 669)	−0·72	
5*a*	—	6·51	6·45 (M 25)	−0·06	L 665: about 6·4
b	0·10	6·10	6·15 (M 25)	+0·05	L 665: 6·1
c	0·25	5·42	5·5 (L 665)	+0·08	
d	0·50	4·72	4·6 (L 662)	−0·12	
e	0·10	6·91	>6·6 (M 25)	*c.* −0·2	L 665: >6·7; L 669: about 6·6
f	0·20	7·25	6·9 (L 669)	−0·35	
g	0·30	7·75	7·1 (L 669)	−0·65	

Sample *a*: without addition of acid or alkali.
Samples *b*, *c* and *d*: addition of lactic acid (1 *N*).
Samples *e*, *f* and *g*: addition of NaOH (1 *N*).
Estimation of *p*H after half an hour's storage at room temperature.

Table 3. *Milk and whey of cows suffering from mastitis*

a = milk; *b* = whey.

No.	mg. % of chlorine*	*p*H				
		° C	Glass electrode	Lyphan M 25	Difference	
1*a*	257	17·6	6·82	>6·6	*c.* −0·2	L 669: <6·6; L 665: no comparison possible
b	—	17·6	6·76	*c.* 6·6	*c.* −0·1	L 665: about 6·7; L 669: <6·6
2*a*	370	17·6	6·19	6·2	+0·01	Not sufficient to prepare whey
3*a*	326	19·2	7·19	>6·7 (L 665)	*c.* −0·4	L 669: 6·7; no rennet coagulation
4*a*	189	21·6	6·81	>6·6	*c.* −0·2	L 669: 6·6
b	—	21·6	6·80	>6·6	*c.* −0·2	L 669: <6·6; incomplete rennet coagulation
5*a*	117	19·0	6·73	>6·6	*c.* −0·1	L 669: <6·6
b	—	19·0	6·68	>6·6	*c.* −0·1	L 669: about 6·6
6*a*	181	19·0	6·97	>6·6	*c.* −0·3	L 669: 6·7
b	—	19·0	7·12	>6·6	*c.* −0·5	L 669: 6·6; incomplete rennet coagulation
7*a*	104	19·0	6·72	>6·6	*c.* −0·1	L 669: <6·6
b	—	19·0	6·74	>6·6	*c.* −0·1	L 669: <6·6; incomplete rennet coagulation
8*a*	136	19·0	6·82	>6·6	*c.* −0·2	L 669: <6·6
b	—	19·0	6·84	*c.* 6·6	*c.* −0·2	L 669: <6·6
9*a*	96	19·0	6·56	*c.* 6·6	*c.* +0·04	
b	—	19·0	6·58	6·6	+0·02	L 669: <6·6
10*a*	114	18·0	6·66	6·6	*c.* −0·06	L 669: <6·6; L 665: no comparison possible
b	—	17·4	6·62	6·6	−0·02	L 669: <6·6; L 665: 6·7
11*a*	170	18·0	6·77	>6·6	*c.* −0·2	L 669: <6·6; L 665: no comparison possible
b	—	17·4	6·79	>6·6	*c.* −0·2	L 669: 6·6; L 665: 6·7
12*a*	139	18·0	6·83	>6·6	*c.* −0·2	L 669: <6·6; L 665: no comparison possible
b	—	17·4	6·82	>6·6	*c.* −0·2	L 669: 6·6; L 665: 6·7
13*a*	107	18·0	6·70	>6·6	*c.* −0·1	L 669: <6·6; L 665: no comparison possible
b	—	17·4	6·67	6·6	−0·07	L 669: <6·6; L 665: 6·7
14*a*	147	18·0	6·70	6·6	−0·1	L 669: <6·6; L 665: no comparison possible
b	—	17·4	6·68	6·6	−0·08	L 669: 6·6; L 665: 6·7

* Chlorine values in normal milks ranging from about 90 to 130 mg.% (mg. in 100 cm.³). As a rule chlorine values are increased in mastitis.

Discussion of results

The results are shown in Tables 1–3.

Besides the samples of milk mentioned in Table 1, a further series of thirty samples was examined, viz. three groups of ten samples, after 4 hr., 28 hr. and 52 hr. storage at 22° C. The *p*H values determined by means of the glass electrode were found to range between the limits mentioned in Table 1, the average *p*H values being 6·62 (18° C.), 6·36 (18° C.) and 4·52 (18–20° C.) respectively. Average values for the titratable acidity were 7·75, 9·06 and 34·29 respectively.

The samples of the latter series stored for 4 and 28 hr. were tested with L 665 (*p*H 5·2–6·7): no comparison was possible on account of difference of colour (the "un- known" being more blue, as compared with the colour scale being more violet). The samples stored for 52 hr. were tested with *L* 662

(*p*H 3·9–5·4), eight samples showing a deviation of −0·1 *p*H and two samples a deviation of −0·2 *p*H.

In the *p*H range 5·4–6·6 only M 25 can be used. In the first thirty-seven values for normal and acid milk mentioned in Table 1, one sample showed a deviation of −0·2 *p*H. In the remaining thirty-six samples the difference of *p*H did not exceed 0·1 *p*H. In most cases lyphan paper indicated lower *p*H values.

In alkalinized milk (*p*H 6·9–8·9) the three papers used, viz. L 669 (*p*H 6·6–8·1), L 671 (*p*H 7·5–8·7) and L 674 (*p*H 8·2–9·7), showed differences up to −1·2 *p*H, so that lyphan paper cannot be used to test alkalinized milks.

Table 2 shows that for normal and slightly acid whey the agreement between the two methods when using M 25 is also good. The deviation does not exceed 0·2 *p*H. In strongly acid whey two deviations of −0·34 *p*H occurred. In alkalinized whey the agreement was so bad that lyphan paper could not be used.

Table 3 shows that lyphan paper can only be used for testing milk and whey originating from cows suffering from mastitis when the *p*H does not much exceed the normal value 6·6. (As a rule the deviation does not exceed −0·2 *p*H.) At *p*H *ca.* 7 and above, the agreement between the colorimetric and the potentiometric measurements is not good, viz. $> -0{\cdot}3$ *p*H. As a rule M 25 furnishes the best results.[1]

SUMMARY

Lyphan paper M 25 can be used for *p*H measurements in milk and whey in the *p*H range 5·4–6·6. As a rule the error does not exceed 0·1 *p*H.

In very acid milk and whey (*p*H < 5·4) lyphan paper of the L series may be used. Generally the deviation does not exceed −0·2 *p*H. In alkaline milk and whey lyphan paper cannot be used.

In milk and whey from cows infected with mastitis M 25 may be used unless the *p*H exceeds 7.

I am indebted to Mr H. Hooghoudt for technical assistance.

REFERENCES

(1) ASCHAFFENBURG (1938). *J. Dairy Res.* **9**, 336.
(2) SEEKLES (1936). *Biochem. Z.* **288**, 402.

[1] *Note during correction of the proof.* Four samples of milk originating from cows suffering from mastitis were tested with *a new lyphan paper* M 35 (*p*H 6·6–8·1). Values obtained: glass electrode (20° C.) 7·17, 7·03, 7·10 and 6·50 respectively; M 35 7·3, 7·2, 7·5 and 7·2 (ca. 6·6 with M 25) respectively. The agreement between the two methods when using M 35 is not always good.

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BIENNIAL REVIEWS OF THE PROGRESS OF DAIRY SCIENCE

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SECTION C. DAIRY CHEMISTRY

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INTRODUCTION

THIS review deals with the progress of dairy chemistry from the middle of 1937 to the middle of 1939. This period is characterized by the wide variety of the subjects that were extensively and profitably studied.

I. COMPOSITION OF MILK

(*a*) COW'S MILK

During recent years it has become increasingly evident that the frequency of occurrence of milk containing less than the presumptive standard of 8·5% solids-not-fat has been underestimated, and this has resulted in greater attention being given to the causes of variation in the solids-not-fat content of milk. At the beginning of the period covered by this review, Davies(1) discussed the composition of milk with a low content of solids-not-fat and pointed out that although udder disease, end of lactation, and hot dry summer weather tend to reduce this content, many apparently

healthy cows, under good management, produce milk with an abnormally low solids-not-fat content throughout lactation. This constituted a problem of obvious importance from the nutritional, commercial and legal aspects, and as its cause was not appreciated remedial measures were lacking.

Later, during an investigation of the protein distribution in milk with a low content of solids-not-fat, Rowland(2) made the suggestion that mild subclinical mastitis might account for a high proportion of the widespread cases of low solids-not-fat. This suggestion was confirmed by Foot & Shattock(3) who, on two occasions, investigated the incidence of mastitis in over a hundred cows which had recently yielded milk containing less than 8·5% solids-not-fat at two consecutive milkings. Bacteriological tests showed that on the first occasion 55%, and on the second 68%, of the animals were suffering from mastitis (*Str. agalactiae*), but Foot & Shattock gave sound reasons for considering that these figures for the incidence of infection were low.

Rowland & Zein-el-Dine(4) continued the study of the effect of subclinical mastitis on the solids-not-fat content of milk by comparing the solids-not-fat percentage of the milk from the individual infected quarters of sixty-two cows in full milk with that from their normal quarters, 121 out of a total of 247 quarters being infected. To avoid differences which might be due solely to the presence of varying amounts of fat, the solids-not-fat content was expressed as a percentage of the fat-free milk. It averaged 9·36, 9·11, and 9·82% in the milk samples from the uninfected quarters of the Shorthorn, Friesian, and Ayrshire cows respectively, but in the corresponding infected quarters only 8·44, 8·38, and 8·28%. These figures show clearly the effect of subclinical infection. Further classification of the data showed that 63% of the infected samples were below 8·80%, averaging 7·93%, but that only 9% of the samples uninfected with *Str. agalactiae* were below this standard, averaging 8·56%. Hence, 88% of the samples low in solids-not-fat were infected samples. The conclusion was reached that subclinical mastitis definitely accounts for a very high proportion of samples of milk persistently low in solids-not-fat content, and produces a large decrease in that content.

The papers outlined above have demonstrated that the first step towards the improvement of a condition of low solids-not-fat should be the control of latent mastitis. Changes in feeding and management would be of no avail when unsuspected mastitis can have so pronounced an effect.

From an investigation of the variation of the composition of the milk of the Missouri Station herd, Herman(5) deduced that season and temperature together account for the known lower solids-not-fat content of the milk of summer months. Whereas the range of solids-not-fat over 12 months was 8·0–9·7%, for July to September it was 8·1–8·7%. Trambics(6), who examined bulk Hungarian milk twice per month for four years, also observed the summer decrease in solids-not-fat and found it to be most marked in very hot summers.

Authoritative figures for the average fat content in the milk of cows of the Ayrshire, British Friesian, Guernsey, Jersey, Lincoln Red, Red Poll and Shorthorn breed are included in the reports of the milk-recording section of the Ministry of Agriculture's livestock improvement scheme(7,8). The latest published figures (for the year 1936–7) are 3·81, 3·29, 4·61, 5·05, 3·72, 3·64 and 3·62% respectively. The variations at consecutive milkings in the fat content of milk from herds of small numbers of cows have been investigated by Erbacher(9) as a guide to the reliability of samples taken for control purposes. He included the effects produced by various changes in the management of the cows. Jeschki(10) studied the variations in the composition of milk from individual cows, and tabulated data for the specific gravity, fat, solids-not-fat, chloride, refractometer number and freezing-point for

the milk of each of six cows examined frequently during several months. Carrying refinement a step farther, Scholz(11) determined the variations in composition when the milk of three cows was drawn in four separate stages at each milking.

Smith *et al.*(12) followed the changes in the fat, solids-not-fat, protein, lactose and chloride contents of the milk of three cows during a 12-day fast. The protein and fat contents rose considerably, the chloride content increased, and the lactose content decreased. It was observed that the sum of the protein, lactose and ash constituents was less than the solids-not-fat content by, at times, as much as 0·19%, and the authors postulate the presence in the abnormal milk of an unidentified constituent, the nature of which has not been elucidated. Reder(13), in an attempt to throw light on the intermittent production of milk with a rancid off-flavour by certain of the animals in a Jersey herd, compared the chemical composition and properties of the normal and rancid milk samples. She found that the milk from cows which were liable to produce rancid samples had a higher chloride and lower lactose content than normal milk produced at the same period of lactation; higher fat, total solids, protein, and lipase contents; lower *p*H and higher titratable acidity.

The changes which take place in the composition of milk as a result of mastitis infection form the basis of various simple indirect tests for the diagnosis of the disease. Munch-Petersen(14) has reviewed the literature concerning these tests, and the reliability of some of them has recently been examined against accepted direct bacteriological methods by Davis & McClemont(15) and Rowland & Zein-el-Dine(16). The following tests were placed in descending order of value for diagnosis thus(16): chloride content, centrifuge deposit, solids-not-fat content, bromcresol-purple reaction, brom-thymol-blue reaction, presence of clots, and catalase content. It is surprising that Johns & Hastings(17) should find the catalase test preferable to the chloride test. However, the natural variation in the composition of normal milk makes it impossible to fix, for the interpretation of simple indirect tests, standards which will prevent them from failing to detect a large proportion of the infected quarters and, at the same time, from diagnosing many uninfected quarters as infected. These tests must be considered most unsatisfactory. Even a combination of two or more of them, although giving a lower proportion of missed positives, gives a higher proportion of false positives(15) and this constitutes an effective answer to those who continue to use them for eradication purposes. Davis & McClemont(18) have evolved a simple test which gives a reasonably accurate indication of milk samples which are of a composition sufficiently abnormal to render them unsuitable for cheesemaking. It is a combined brom-cresol-purple-rennet test, and hence is influenced by abnormality in the reaction and in the calcium, casein, and soluble protein contents of the sample.

(*b*) Milk of other species

Goat's milk

The scarcity of reliable information on the composition of the milk of the goat under English conditions has been remedied in a valuable paper by Knowles & Watkin(19), who give data covering two complete years for 345 animals in ninety-nine herds, comprising eight different breeds. The effect of breed, stage of lactation and age of the goat on milk composition is indicated, and the following average composition deduced: fat 4·50, solids-not-fat 8·68, lactose 4·08, total protein 2·90, casein 2·47, soluble protein 0·43, ash 0·79, CaO 0·194, P_2O_5 0·270, and chloride 0·154%. Gamble *et al.*(20) have made a comprehensive study of goat's milk in America, and provide these averages: fat 3·5, total solids 11·71, protein 3·10, lactose 4·55, ash 0·79%, iron 0·068, copper 0·053, calcium 114 and phosphorus 98 mg./100 ml.

The detailed composition of the protein and fat was also investigated. Their averages are unusual in that the solids-not-fat percentages—the difference between the total solids and fat percentages—is less than the sum of the protein, lactose and ash, whereas one would expect it to be slightly larger. Jeschki(21) records fat 3·8%, solids-not-fat 8·4%, as the average of forty-four samples of goat's milk. The energy value of goat's milk was determined by Peterson & Turner(22) for various stages of lactation. They gave the energy value of 1 lb. of milk of 4% fat content as 342·3 cal., and found that this value could be calculated from the total solids, fat, protein or lactose percentages.

Mare's milk

Linton(23) has continued his investigations on mare's milk with a study of the variations during lactation in the percentages of total solids, fat, solids-not-fat, protein, lactose, ash, CaO, P_2O_5, and chloride, and in the freezing-point and *p*H. As lactation advanced, the percentages of total solids, fat and lactose increased, whereas solids-not-fat, protein, CaO and P_2O_5 decreased.

Miscellaneous

The milks of many different mammals, with composition ranging from 65–88% of water, 2·0–6·7% of lactose, and 0·23–2·5% of ash, have been demonstrated by Sharp(24) to show a definite relationship between their lactose and ash contents when both are calculated as g./100 g. of water. The percentage composition of a sample of elephant's milk was reported by Nottbohm(25) as water 73·15, fat 15·12, protein 4·89, lactose 3·38, ash 0·76 and total solids 26·85. The lactose figure was only half the values previously recorded, but these were obtained by difference and the present figure by direct measurement.

II. CHEMISTRY OF MILK CONSTITUENTS

(*a*) Milk fat

Composition

Further attention has been paid to the component fatty acids of butterfat by Hilditch & Longenecker(26), who, by using an electrically heated and packed column for the distillation of mixtures of volatile esters, isolated fractions with very high proportions of unsaturated esters of low molecular weight, including decenoic, dodecenoic, tetradecenoic and hexadecenoic acids. Oleic and octadecadienoic were the only C_{18} unsaturated acids present. Bromine additive products indicated the presence of arachidonic acid, and the following saturated acids were identified: octanoic, *n*-decanoic, lauric, myristic, palmitic and stearic. The total acids of the sample of butterfat examined were calculated to be as follows: butyric 3·03, *n*-hexanoic 1·39, *n*-octanoic 1·51, *n*-decanoic 2·68, lauric 3·71, myristic 12·01, palmitic 25·17, stearic 9·17, arachidic 1·32, decenoic 0·32, dodecenoic 0·42, tetradecenoic 1·58, hexadecenoic 3·96, oleic 29·54, octadecadienoic 3·57, and arachidonic 0·28%. In the lower unsaturated acids whose presence has been discovered the ethenoid linkage was found to occupy the $\Delta^{9,10}$ position, and this is suggestive in connexion with the theory that the lower fatty acids in milk fat are produced from oleic groups already existing as glycerides. The minor unsaturated components may represent fragments of transformed oleoglycerides which have escaped complete saturation to lower saturated glycerides.

The same theory receives support from Smith & Dastur's(27) work on the effect of inanition on the composition of milk fat, as about 80% of the lower fatty acids

were replaced by oleic acid during starvation of the cow. Starvation caused marked progressive changes in the nature of unsaturated acids, and a marked decrease in that of lower fatty acids normally present. The butyric and myristic acid contents fell from 3·5 to 1·2% and 11·9 to 2·8% respectively, and the sum of all acids up to and including C_{14} fell from 22·6 to only 4·8%, whilst the oleic acid content rose from 35·9 to 52·8%. On a molar basis a reduction of 24·2 molecules of lower acids was mainly accounted for by an increase of 19·6 molecules of oleic acid.

The seasonal variations in the saponification, iodine and Reichert-Meissl values of butterfat were discussed by Torfs(28), and a number of papers on the effect of various rations on the constants of butterfat(29,30) bear out existing data that the butterfat assumes some of the characteristics of the fat or oil of the food.

Fat oxidation

Oxidized flavour in milk continues to receive much attention, for outbreaks occur when metal contamination and exposure to sunlight, with their catalytic effect, are known not to be the cause. Only a certain number of the individual cows in a herd are liable to produce milk that is susceptible to the development of oxidized flavour, and for this reason attempts have been continued with some success to correlate the composition of milk with its tendency to off-flavour development, the possible anti-oxygenic properties of the vitamins, the lipides, and carotene being chiefly concerned. Also a further series of experiments has extended our knowledge of the important effect of the feed of the cow on the development of oxidized flavour, but less progress has been made in the elucidation of the source and mechanism of the reactions causing the trouble.

Garrett *et al.*(31) found a significant correlation of carotene content and of ascorbic acid content with good flavour in the milk from individual cows. Dahle & Palmer(32) and Kieferle & Seuss(33) showed that the addition of ascorbic acid to milk prevented or lessened the development of oxidized flavour. Beck *et al.*(34), on the other hand, from an examination of the vitamin C content of over 700 milk samples from four breeds, concluded that no relationship existed between this content and susceptibility to oxidized flavour. The lecithin content of the milk likewise showed no correlation, but there was an inverse relationship between the depth of colour of the milk fat and its tendency to taint. In 480 samples from Channel Island breeds only 6% gave oxidized flavour, while in 448 samples of Holstein and Ayrshire milk 17·4% yielded flavours. Trout & Gjessing(35) have recently commenced a study of ascorbic acid and oxidized flavour in milk.

In general, green foods prevent and winter rations promote oxidized flavour, the important factors being the anti-oxidants ascorbic acid and carotene. Dahle & Palmer(32) report that the feeding of tomato or lemon juice, ascorbic acid, alfalfa or carrots inhibit oxidized flavour. Brown *et al.*(36) also found this effect on the addition of lemon juice, ascorbic acid, or carotene to the cow's ration, the carotene being the more effective. The carotene supplement caused a definite rise in the carotene content of the milk, and a slight rise in the vitamin C content; the ascorbic acid supplement caused a slight rise in both. Cave *et al.*(37) showed that the feeding of vitamin A did not reduce the incidence of oxidized flavour and that the feeding of carotene eliminated it. Hening & Dahlberg(38) found that the feeding of mangels or dried beet pulp "in no way prevented or increased the susceptibility of milk to the development of oxidized flavour". This is not surprising, as both are of very low carotene content.

The actual mechanism of the development of oxidized flavour still remains a problem. It occurs in milk whilst the true butterfat is still in the very early stages of

its induction period, and it appears that other substances are important in the early stages of oxidation. An extensive and valuable study has been made by Dahle & Palmer(32), who have investigated the suggestion made earlier by Thurston that oxidized flavour is caused by oxidation, not of the butterfat, but of the phospholipides of the fat globule membrane. They showed that those phospholipides are highly unsaturated and produce oxidized flavour rapidly, but that there are other factors in this flavour development which need explaining. For example, heating milk to 168° F. or above, and cooling immediately, prevented the flavour development, but heating at 145° F. for 30 min. or 160° F. for 5 min. gave a strong flavour. The effect of the high-temperature treatment might be explained in two ways, either the destruction of a causative factor for flavour production or the liberation of reducing substances which tend to prevent it. The effect of the low-temperature treatment, likewise, may be due to the destruction of preventive bacteria which act as reducing substances. In this connexion the possible destruction of vitamin C—a reducing substance—must be remembered. Dahle & Palmer have shown experimentally that a causative factor is present in the serum of samples of milk or cream which are susceptible to off-flavour, and have revived the enzyme (oleinase) theory of Kende to account for it. They have, however, complicated the above observations by demonstrating that it is possible to produce oxidized flavour in milk prepared in such a way that its fat globules are free of the usual phospholipide membrane provided the causative factor (enzyme) from susceptible skim milk be present. If non-susceptible skim milk was used in making this milk, then even the addition of copper salt failed to produce off-flavour. Swanson & Sommer(39) have given a brief description of experiments tracing the oxidized flavour of milk to fat-related substances, and not to the butterfat. They also considered that the oxidation-reduction potential of the milk was not a factor in flavour production, thereby conflicting with Greenbank(40), who deduced that the inhibition of flavour development by high-temperature heating or the feeding of green foods was due to a lowering of the oxidation-reduction potential.

In a study of the chemistry of incipient oxidation defects in butter, Barnicoat & Palmer(41) have been concerned with the off-flavours, variously known as flat, bitter, stale storage and metallic, which are promoted by oxidation catalysts and may occur long before there is any evidence of oxidation in the butterfat. From their bulletin it would appear that oxidation of the lecithoprotein surrounding the fat globules is responsible for the incipient off-flavours. Vitamins C and E, and carotene, were shown to be antioxygenic, but the antioxygenic effects of the soluble phosphates and citrates of the butter serum were more striking. These substances were previously known to be antioxidants for other fats. Barnicoat & Palmer put forward the following theory for the production of oxidation taints. In milk or cream, according to modern concepts of emulsification, the lipide portion of the lecithoprotein complex is in the fat globule, the protein portion extends into the aqueous phase. In the presence of dissolved air, traces of adsorbed metals (probably as metaproteinates) catalyse the oxidation of the lecithoprotein which may later transmit the oxidation to the triglycerides in the fat globules. After churning to butter the fat might continue to oxidize until the oily or tallowy flavours arising from the oxidation of unsaturated acids appear, but with ordinary well-made butter these troubles are now quite uncommon, and in all probability, by the time the butter is consumed, oxidation, if started, will have advanced only so far as to promote a "stale-storage" flavour. Barnicoat & Palmer, therefore, differentiate between incipient off-flavours and the pronounced tallowy defects which are common to many fats, and readily detectable by chemical methods.

(*b*) Proteins

The solubility of casein in sodium benzene-sulphonate, sodium cymene-sulphonate and potassium thiocyanate has been determined by McKee & Gould(42), who showed, for the first time, that solutions of the sulphonates at *p*H 4·6 will separate casein into at least two components differing in phosphorus content, formaldehyde-absorbing capacities, and solubility in pyridine solution. These two nearly neutral sulphonates obviate any hydrolysis during fractionation, and the experiments accord with the existing evidence that casein is a mixture of related components. By measurement of its electrophoretic mobility, Mellander(43) found cow's milk casein to consist of three well-defined components. The components of highest and of lowest mobility were isolated, the former had a P/N ratio larger, and the latter much smaller, than that of the total casein.

The action on casein of 1% sodium hydroxide at 37° C., followed by acidification, in producing a precipitate hitherto called dephosphorized casein has been further investigated by Plimmer & Lawton(44). Casein is converted into two phosphorus-free portions, one the precipitate and the other in solution. The precipitate contains 55% of the nitrogen of the original protein, resembles a metaprotein in its solubility in dilute acid and alkali, and resembles casein in being precipitated by more acid. The precipitate is a dephosphometacasein, for which the term dephosphocasein, or briefly depocasein, is proposed. The protein in solution resembles a primary proteose, and is termed depocaseose. The amino-acid composition of depocasein is given; in general it is similar to that of casein.

Realizing that analyses of the amino-acids present in the casein and albumin of cow's milk had frequently been made, but that analyses of the corresponding proteins of human milk were lacking, Plimmer & Lowndes(45) prepared and analysed the casein and lactalbumin (actually heat-coagulated lactalbumin and lactoglobulin) of both kinds of milk to obtain comparative figures. Data are given for their nitrogen, sulphur and phosphorus contents, Van Slyke nitrogen distribution, and arginine, histidine, lysine, tryptophane, tyrosine, cystine and methionine contents. By expressing the various amino-acids as percentages of average human and cow's milk, it was shown that cystine is present to the same extent in these milks, and that cow's milk contains two to three times the amounts of the other amino-acids. Later, Beach *et al.*(46) compared the amino acid contents of human and cow's milk and obtained figures in broad agreement with Plimmer & Lowndes. Kassell & Brand(47) applied a reliable system of analysis to the sulphur distribution in casein and lactalbumin, and showed that all the sulphur in these proteins can be accounted for as cystine, cysteine, and methionine.

A flavoprotein has been isolated from cow's milk by Corran & Green(48). The purest preparation obtained yielded 0·12 g. from 100 l. of milk, which, on an estimated loss of 95% in the purification processes, suggests 2·4 g. of flavoprotein per 100 l. as the order of magnitude. Solutions of this protein at concentrations greater than 10 mg./ml. were orange-red in colour. The physical and chemical properties of the flavoprotein and its prosthetic flavin group were described. Grimmer & Geidel(49) have isolated a protein, which is apparently combined with lipides, from the alcohol-soluble portion of cream separator slime. Turnau(50) has drawn attention to the different values obtained by various workers for the percentage of nitrogen in the milk proteins.

The demonstration of the presence of secondary proteins of a proteose-peptone nature in fresh milk has been followed, by Rowland(51), with the application of improved methods of separation to the proteins of normal and abnormal milk. The results so obtained provide the first full data on the relative proportions of casein,

albumin, globulin, proteose-peptone, and non-protein nitrogenous substances in milk. The average nitrogen distribution in normal milk was as follows: casein 78·5, albumin 9·2, globulin 3·3, proteose-peptone 4·0, and non-protein 5·0% of the total nitrogen. On this basis normal milk contains, as an average, casein 2·63, albumin 0·31, globulin 0·11 and proteose-peptone substances 0·13%, giving a total protein content of 3·18%. Casein here accounts for a higher percentage of the total nitrogen than has been found in previous investigations. This is explained partly by the use of a method ensuring more accurate casein precipitation, and partly by the avoidance, where information was available, of milk from cows with mastitis. Mastitis-infected milk was characterized by a decreased content of casein, and an increased content of albumin, globulin, and proteose-peptones, particularly globulin.

A later paper(52) describes the results of the determination of the total and casein nitrogen contents of the milk from the 247 individual quarters of sixty-two cows in full milk; the quarters were examined bacteriologically for streptococcal mastitis, 121 being found infected. The total nitrogen content of the samples varied from 362 to 687 mg./100 ml. and the casein nitrogen from 153 to 554 mg./100 ml. It was observed that in the milk secreted by healthy quarters, although the content of total nitrogen varied over a wide range, the casein accounted for an almost constant percentage of that total nitrogen. This percentage was termed the "casein number". It averaged 79·2, 80·4, 79·5 and 80·3 in the samples from uninfected quarters of the Shorthorn, Friesian, Ayrshire and Guernsey breeds respectively, and only 71·7, 74·1 and 70·3 in infected samples from the first three breeds. Covering all samples, the casein number ranged from 42·3 to 83·4. The most interesting result of this work was the introduction of the casein number as a useful chemical method for the diagnosis of mastitis. When a figure of 78·0 and less was taken as indicating an infected quarter, the proportion of instances which disagreed with the bacteriological method was only 8·6%.

While investigating the genetical aspect of the protein content of milk, Azarme(53) found that the percentages of total, casein, and soluble protein nitrogen decreased markedly from the beginning to the fourth week of the lactation period and then increased slowly till near its end, when the rise became rapid.

(*c*) Lactose

Investigations strictly on the chemistry of lactose have been lacking during the period under review.

(*d*) Mineral matter

Calcium, magnesium and phosphorus

Lampitt *et al.*(54) have continued their study of the constituents of milk by means of dialysis, and added magnesium to the list of constituents determined. This is a welcome extension, as the available data for magnesium (compared with calcium and phosphorus) is indeed meagre, and this study is noteworthy for the great care taken in assuring the reliability of every analytical method used. Lampitt *et al.* have noted the effect on the content of dialysable calcium, magnesium, inorganic phosphorus and organic phosphorus of acidification, acidification followed by neutralization, and extended agitation. Among the many interesting results in need of explanation were the following: An approximate straight line relationship exists between the concentration of dialysable calcium and inorganic phosphorus of milk powder solution and the titratable acidity at the acidification stage, but neutralization of the added acid does not result in the original amounts of these constituents being

regained. The amount of dialysable organic phosphorus is not affected by these treatments. The neutralization of acidified raw separated milk results in an almost complete recovery of the original amounts of dialysable calcium, magnesium and inorganic phosphorus, although the figure for calcium remains slightly above normal. Extended agitation has been found to reduce the amounts of dialysable calcium and magnesium, and to increase the amount of dialysable inorganic phosphorus in raw separated milk. Agitation has no effect on the constituents of milk-powder solution, unless it has been acidified and neutralized. The following range of analytical figures for average raw, pasteurized, separated and dried milks supersedes those the authors have given previously:

	Total %	Dialysable as % of total
Inorganic P	0·63–0·77	33–43
Organic P	0·31–0·38	7–15
Calcium	1·27–1·44	25–42
Magnesium	0·10–0·15	62–83

Ling(55) has used his titration method for the estimation of tricalcium phosphate in milk to explore the obscure partition of calcium and phosphorus. His lengthy paper gives much useful data which is difficult to summarize. The partition of the total calcium may be represented approximately as one-third soluble and two-thirds colloidal, two-thirds of the colloidal being tricalcium phosphate and the remainder casein calcium. Phosphorus is partitioned into soluble and insoluble organic and inorganic. The relationship between casein and mineral constituents is discussed, the evidence of the work supporting the view that casein is associated with tricalcium phosphate in a physical manner.

Chloride

An extensive analysis of the chloride content of milk has been made by Davis(56), its variation with breed, stage of lactation, age of cow, season, disease, etc., being included. Frequency distributions are tabulated for both the a.m. and p.m. milk of individual cows of the Shorthorn, Friesian and Guernsey breeds. The modes of the frequency distributions were in the 71–80 mg.% range for Shorthorn and Guernsey milk, and the 111–120 mg.% range for Friesian milk. It must be pointed out that the Friesian data cannot be accepted as representative, as the herds were known to produce milk of unusually low solids-not-fat content and to suffer from mastitis, so that high chloride figures must be expected. Evening milk showed a considerably lower chloride percentage than morning milk, 5 mg.% less in the Guernsey milk and 8 mg.% in the Shorthorn and Friesian. The variations in the sodium content of milk during the lactation period are described by Vuk & Sandor(57), who give the average figure of 0·296 g./l. for cow's milk, 0·398 for sheep's milk, and 0·256 for goat's milk.

Trace elements

Dingle & Sheldon(58) and Drea(59) have made spectrographic examinations of human and cow's milk. It is difficult to obtain electrodes sufficiently free from certain of the elements likely to be present in milk in traces, and this may account for the discrepancies in their results. Certainly their findings must be viewed cautiously. For example, Dingle & Sheldon concluded that manganese is a normal constituent of both milks, whereas Drea found no sign of it in cow's milk, and detected it in only three out of six samples of human milk. Drea found chromium to be present, Dingle & Sheldon did not. Drea detected copper and iron, but Dingle & Sheldon, surprisingly enough, considered their presence doubtful. A comprehensive review of many papers dealing with the lesser minerals of milk has been written by Tellmann & Frehden(60).

(*e*) Enzymes

Lipase

The presence in fresh clean milk of a preformed triglyceride-splitting enzyme has been verified by Mattick & Kay(61), who, rightly, were not satisfied that previous work had been sufficiently rigidly controlled to obviate lipolysis by micro-organisms or by enzymes secreted by them. A reliable method has been developed for the estimation of lipase (tributyrinase), and its occurrence and relationship to the lactation cycle have been studied. The lipase concentration in milk varied considerably from cow to cow, and in healthy cows there was a fairly regular variation during lactation. The lipase content was high in colostrum, decreased rapidly in the next few days, and then rose to a value which did not vary much until the very end of lactation, when a slight decrease occurred. This was unexpected, as the literature contains papers which refer to a definite increase at this time. Herrington & Krukovsky(62,63,64) have also studied the lipase of milk. They determined the extent of lipolysis in milk by titrating the acidity of the clarified fat obtained after churning the milk. This cannot be considered a measure of the true extent of lipolysis, as the liberated fatty acids would be distributed between the fat and the serum of the milk. Most milk samples showed the presence of a formaldehyde-sensitive and a formaldehyde-resistant lipase. Krukovsky & Sharp(65) have shown that the shaking of raw milk induces lipolysis. This is attributed to an alteration in the surface characteristics of the fat globules, creating a more favourable condition. This lipolysis continues even after the milk has been cooled to a low temperature. Jones *et al.*(66) also measured the lipase activity of milk; they found that it does not necessarily increase throughout the lactation period, and that lipase is not the cause of bitterness in milk.

Phosphatase

During the period under review the phosphatase test for the control of the efficiency of pasteurization has received much attention, including the adaptation of Kay & Graham's test to pasteurization temperatures lower than 145° F., and the introduction of modifications involving the actual technique. The adaptation to lower temperatures has been tried in two ways; either shortening the time of incubation and retaining the colour standard of 2·3 Lovibond blue units, or retaining the original incubation time of 24 hr. and using a higher colour standard. Krueger(67) obtained satisfactory results with an incubation time of 8 hr. for milk pasteurized at 144° F. for 30 min. and Smith(68) with 4¼ hr. for 143° F. Storrs & Burgwald(69) experimented with various temperatures from 140 to 145° F. and incubation times of 2½, 5 and 24 hr. Their results, like those of Burgwald & Giberson(70), show unusually low blue colour readings, not in accord with other work. This suggests some abnormality in technique.

The reduction of the incubation time for milk pasteurized below 145° F. reduces the sensitivity of the test to the presence of raw milk, and hence Gilcreas retained the 24 hr. incubation and, for milk pasteurized for 30 min. at 143° F., recommended(71,72) a liberated phenol limit of 0·05 mg./0·5 ml. of milk.

An important modification of the technique of the phosphatase test has been introduced by Scharer(73,74,75); the disodium phenyl phosphate substrate was used, but a borate buffer replaced the barbiturate, the milk proteins were precipitated with basic lead acetate, and Gibb's phenol reagent, 2·6-dibromoquinone chloroimide was preferred to Folin's reagent. An accurate laboratory method and a shorter test were described, with incubation times (for milk pasteurized at 143° F.) of only 1 hr. and 10 min. respectively. Scharer's tests thus save considerable time.

The phosphatase test has been applied to dairy products other than milk (72,75,76,77), but to place these tests on a satisfactory basis careful laboratory investigations are still required.

Peroxidase, etc.

Finck (78) has found the guaiacum reagent to be less sensitive than a *p*-phenylenediamine magnesium borate mixture in the detection of milk peroxidase. Complete destruction of peroxidase, on heating for 5, 10, and 20 sec., occurred at 82, 81 and 80° C. according to the guaiacum test, and at 83, 82 and 81° C. respectively as judged by the amine test. The rate of destruction of the enzyme over the range 74–83° C. was a linear function of temperature. Schwarz & Fischer (79) have prepared from buttermilk a Schardinger enzyme concentrate which was 200–500 times more active than whole milk. The colouring matter of the enzyme was freed from the protein which forms an integral part of the enzyme, and shown not to be identical with lactoflavin, hemin or carotene.

(*f*) Other constituents

For the determination of the phosphatide content of milk, Rewald (80) first prepared whole-milk powder. He found the phosphatide content to be 0·24% in milk powder, equivalent to 0·0314% in fresh milk. Arup (81) has reported 0·150–0·206% for the range covered by the citric acid content of fresh milk. The results for samples of condensed and dried milk from various sources, when calculated in terms of normal liquid milk, agreed with a citric acid content of from 0·151 to 0·205%.

III. PHYSICAL CHEMISTRY AND PHYSICS

(*a*) Milk and general

Temple (82) has described a new apparatus for the rapid and economical determination of the freezing-point of milk, and Davies (83) has discussed the freezing-point test in its general applications. Gangl (84) has also described a new freezing-point apparatus which works almost automatically and prevents errors due to excess supercooling. Determinations are made in less than 2 min. Pyenson and Dahle (85) have studied the importance of bound water in various dairy products. The bound water is calculated from the freezing-point measured by means of Newton and Gortner's cryoscope. The increase in the specific gravity of milk which takes place on ageing (the so-called Rechtnagel phenomenon) is believed to be partly due to an increase in the amount of bound water, as are also various changes in the "body" of cream induced by temperature treatments.

Niemczycki & Galecki (86) have described a new apparatus for determining the electrical conductivity of milk, which is essentially a modification of Gerber's method. The possibility of detecting mastitis by means of conductivity measurements is discussed.

Aschaffenburg (87) has applied a modification of the Lange (gold sol) test for cerebro-spinal fluid to milk serum, and has shown that, in preliminary experiments at least, the test is hopeful for the diagnosis of subclinical mastitis. A casein-free milk serum is titrated with NaOH to a *p*H in the region of 6·0–6·5. When 5 ml. of a red gold sol, prepared in accordance with Lange's directions, are mixed with 1 ml. of this serum, the colour of the gold sol remains unaffected by sera from milk of healthy individual cows or quarters, but turns instantaneously to violet or blue in the case of even slight infection of the udder. The question of the nature of the chemical substances responsible for this reaction is extremely complex.

Rowland(88) has studied the effects of heat on the creaming power of milk. Heating milk for 30 min. at temperatures below 62° C. increases creaming power, at 62° C. the creaming power is unaffected, and at higher temperatures it becomes progressively reduced. This latter reduction is ascribed to progressive denaturation of albumin and globulin.

Aschaffenburg(89) has applied the Wulff *p*H technique to the colorimetric determination of *p*H in milk and whey, and Mohr & Bauer(90) use a similar method (Kloz "Duplex" method) for starter, cream, buttermilk and butter serum. The latter method gives results within less than 0·1 *p*H unit of the electrometrically determined values, the colorimetric results usually being low. Parsons(91), working with butter serum, found the quinhydrone electrode more convenient than the glass electrode owing to the fragility of the glass and the difficulty of keeping it free from fat.

Mohr & Rittenhoff(92) have compared the quinhydrone and glass electrodes for various dairy products. They claim that errors due to fat interfere with the quinhydrone, but not with the glass electrode. Whittier(93) has shown that the buffer action of citrates in milk is exerted principally in the range in which phosphate and casein buffer most effectively (*p*H 4·2–4·5), and is of slight importance compared with the effects of either of these.

Couprie(94) has described a new potentiometer and electrodes for the practical measurement of *p*H in the dairy industry as a whole.

The use of the Leithe-Müller serum in the refractometry of milk is described by Jax(95).

Frühwald(96) has studied the effect of passing milk between two insulated charged electrodes at potentials up to 200 V. on rennet coagulation and churning properties. The process is claimed to improve firmness of curd and quality of butter.

Jack & Dahle(97) have given a good bibliography of electrochemical investigations. They measured the electrokinetic potential of fat globules in milk. The mobilities were little affected by dilution and temperature changes, but salts produced a marked effect. The sign of the fat globules changed at an iso-electric point of about *p*H 4·3.

Pien & Maurice(98) have studied the relationship between density and composition of milk.

(*b*) Curd

King & Melville(99), using disk and cylinder rotation viscometers, have studied the changes in viscosity of milk under the influence of rennet. The viscosity was calculated from the logarithmic decrement of swing, but, as the authors themselves evidently realized, the system ceased to be even approximately a true fluid before the interesting part of the process was reached, and the viscosity figures in this range have little meaning.

A number of authors have measured the "curd tension" of milk either by Hill's method or by some modification of it. Berggren(100) has related "curd tension" to casein content, and studied the effects of varying quantities of gelatine. She has also described a modification of the Bloom gelometer for use with milk-curd and found good agreement between measurements made with this instrument and by Hill's method. Since the plunger in the Bloom instrument was replaced by a knife, this agreement is perhaps not surprising. Saxl(101) has used a less drastically modified Bloom gelometer, and measured the depression of a plunger with a hard rubber tip into gels of various kinds, including dairy products. He studied both the elastic compression at stresses below that required to break the surface, as well as the rupture conditions themselves.

Kelly(102) has described a method for coagulating milk with pepsin in HCl, and studied the rate of digestion (*in vitro*) by means of the curd-o-meter knife.

Chambers *et al.*(103) have defined a "surface number" which was apparently a measure of curd surface area. Feeding trials with infants showed that this was an index of the relative digestibility of milks.

Scott Blair(104) has described an apparatus for measuring the compression modulus of uncut curd in the cheese vat, the curd being almost a truly elastic solid for small deformations, and Coppen(105) has used this technique extensively to test curd in a cheese factory. She compared these measurements with the subjective opinions of the cheese-maker and showed that, although confident opinions are expressed on very small differences in firmness, the smallest differences which could be correctly appreciated were of the order of 8%. Certain differences which cheese-makers claimed to make in the manufacture of different types of cheese were found to be illusory, and seasonal effects objectively verified were not appreciated by the cheese-makers.

Scott Blair & Coppen(106) have measured the smallest differences in the viscosity of true fluids and the compression modulus of elastic solids that could be distinguished by cheese-makers and others handling the materials, and found that elasticity could be assessed about three times as accurately as viscosity, and that cheese-makers showed no superiority in either test. Scott Blair(107) also discussed the general application of the methods of psycho-physics to the dairy industry.

(*c*) Cheese

Davis(108) has studied the elastic and plastic ("rheological") properties of cheese as well as the plastic properties of butter. By loading cylinders of material and observing the deformations produced, as well as the proportions of these deformations which can be recovered when the loads are removed, he was able to calculate viscosities and elastic moduli in c.g.s. units for arbitrary stress and strain conditions. Abnormal conditions in milk, such as mastitis, were reflected in the rheological properties of the cheese.

Koestler(109) has claimed that there is no simple relation between the hardness and elasticity of Emmental cheese and its chemical composition, but the methods used for determining the former were somewhat inadequately described. Marquardt(110) described the effects of freezing various types of cheese.

Tuckey *et al.*(111) made an X-ray diffraction analysis of Cheddar cheese during a 6 months' ripening period and attempted to follow the formation of amino-acids in the proteolysis, making comparisons with amino-acids extracted with *n*-butyl alcohol. Leucine and iso-leucine were identified at all stages of ripening, and tryptophane, tyrosine and perhaps lysine, in the later stages. Indications of an X-ray spectrum for casein were also claimed.

(*d*) Cream

Skelton & Ellenberger(112) showed that changes produced by heat treatment in "body" of cream were not dependent on changes in fat-globule clumping. They claimed that pasteurization reduced, and heat treatment increased, adsorption on the globule membrane, and that solids-not-fat in skim milk were lowest with heat-treated cream. Centrifuging produced more skim milk from pasteurized than from raw or heat-treated cream.

Getz *et al.*(113) described a method for making whipped cream by dissolving N_2O in cream in a "syphon" under pressure which is subsequently released. Ageing cream diminished whipping capacity, as do most other processes which tend to increase viscosity (such as addition of gelatine or sodium alginate). The process worked quite well with homogenized cream. The distribution of bubble size was more even in whipped cream made by the process than in that made in the usual way.

Guthrie(114) has studied the viscosity of cultured cream (a type of mayonnaise) by means of a MacMichael viscometer. Viscosity was at a maximum at pasteurization temperatures of 155–165° F. It increased with increasing homogenization pressure at least up to 3500 lb./in.^{2}, was affected by homogenization temperature, and increased by the addition of skim milk.

(*e*) Ice cream

Stebnitz & Sommer(115) have extended their work, referred to in the last review on the relative stabilizing effects of sodium alginate and of gelatine. The former had the advantage that it attained maximum whipping capacity immediately after homogenization and cooling to 40° F., and retained a uniform viscosity during ageing. The comparative merits of sodium alginate and gelatine were also studied by Parfitt *et al.*(116), who measured *p*H with both glass and quinhydrone electrodes. The former electrode gave *p*H values from 0·10 to 0·15 unit higher than did the latter. Leighton & Leviton(117) studied the whipping properties of ice-cream mixes. The phenomena described were very complex. Butterfat reduced and sugar increased the amount of overrun. High homogenization pressures also favoured a big overrun.

(*f*) Butter

The Bureau of Agricultural Economics of the U.S. Department of Agriculture has issued official bulletins (1938 and 1939) giving the standards of quality required in U.S.A. for creamery butter. These also include a very useful description of the terms used to describe consistency of butter in that country.

W. J. Davies(118) discussed the influence of texture on the flavour of butter. He stressed the importance of the size of water globules as distinct from total moisture content, and believed that texture depended more on pasteurization technique, and especially on the cooling (analogous to the tempering of metals), than on the method of churning. Slow cooling gave a soft butter, fast cooling a hard and not greasy butter. Cream should be held for about 12 hr. at churning temperature before churning. W. L. Davies(119) discussed similar problems. Parker & Shadwick(120) discussed the problem of butter flavour and aroma in their psychological and chemical aspects.

Knudsen & Sörensen(121) proposed a method for estimating the size of water droplets in butter. A piece of filter paper was dipped in a solution of alcohol, HCl and bromphenol blue. The dried paper was applied to a freshly cut butter surface for 30 sec. The areas turned blue by the water droplets in the butter could be measured and expressed as a percentage of the total area.

A study of the effect of *p*H of cream on butter quality was made by Bird(122), and Parfitt & Brown(123) have investigated the effect of *p*H and season of the year on the keeping qualities of commercial butter. Clayton & Morse(124) claimed that the formation of butter from cream did not involve a true emulsion inversion. The cream O/W emulsion broke, due to the preference of the proteins for the air/liquid rather than for the fat/water interface. The resemblance of butter to a W/O emulsion was fortuitous. The addition of surface-active substances, such as saponin or egg albumen, to cream inhibited or prevented churning, due to the preferential adsorption of the added material in the froth.

Levowitz & van der Meulen(125) discussed theories of churning and suggested that churning was due to the setting free of butterfat from less stable globules, followed by a cementing together of the more stable protected globules into clumps by the free butterfat. In support of this, these authors have produced butter without churning under conditions that favoured a decrease in the stability of the fat globules.

Raven(126) has made a short study of the relationship between body and texture

of butter with flavour, and Weckel(127) has measured the resistance of butter to crushing, slicing and melting, though details of methods are not given. Overman *et al.*(128) have studied the keeping qualities of butter in cold storage. No simple relation was found to exist between quality scores or keeping qualities, and either analytical data or such physical properties as refractive index. Sweet cream butters were the best for initial score as well as for keeping quality, those from self-soured, partially neutralized creams were the worst, while those from starter-ripened, partially neutralized creams were intermediate in quality.

Much work has been done during the period under review on the spreading capacity of butter, especially by Dutch workers. Mulder(129) has given a general and rather qualitative discussion. He pointed out that the relative hardness of butter differs at different temperatures, and that tests should therefore be made at least at two temperatures. Preferably one temperature should be high and the other low. A good deal of the "body" of butter is "false". i.e. butter softens on working, and hardens gradually on being left to stand. This "false body" phenomenon is now generally called "thixotropy", a term originally used to describe a somewhat different phenomenon. In a second paper(130) the same author described the effects of pasteurization, as well as other processes of heating and cooling of the cream, on butter consistency, and in a third(131) a theory of butter structure was proposed according to which chains of fat globules are formed when butter is left to stand, but this structure is broken down on working, thus accounting for "false body". The effect of temperature on hardness is also discussed.

Kruisheer, Herder *et al.*(132) have described experiments in which a metal rod was loaded with increasing weights until it just penetrated the surface of butter in the barrel. The weight which just caused penetration (the yield value) was found to be closely correlated with "firmness" as judged subjectively by the butter grader. The velocity of sinking of the rod through the butter was also measured and the effect of varying temperature was studied. The work has been extended somewhat in a second paper(133).

Pasveer(134) criticized Kruisheer's methods, claiming that butter resembled a fluid rather than a solid, and that "yield value" was therefore meaningless as applied to butter. The yield value which Kruisheer claimed to observe was the result of a number of factors of which the chief was an inability to observe small deformations. Pasveer has also criticized Kruisheer's arrangement for temperature control. He has devised a method of his own in which cylinders of butter are flattened out by dropping a weight from a fixed height.

Kruisheer and his co-workers replied to Pasveer's criticisms(135) by showing that if a true fluid (sugar syrup) is loaded under the same conditions as butter, the smallest loads produce measurable deformations, thus showing a marked difference of behaviour since, as they state, butter is not a true fluid. The whole controversy about yield values might have been avoided had the disputants realized that a yield value is no more than a sharp bend in a flow curve(136), and that many materials are known which are neither true fluids not perfect solids and which show every sort of behaviour from a smooth parabolic relation between stress and rate of shear to a clearly defined yield value below which stress no flow can be observed.

Although butter does not have a constant viscosity independent of stress and strain (i.e. is not a true fluid) a three-dimensional diagram can be constructed relating viscosity to stress and to strain. In this way the plastic properties of butter can be expressed in absolute (c.g.s.) units instead of in terms of arbitrary parameters such as are used by the Dutch investigators. In the investigation already mentioned, Davis(108) has obtained such data by the method devised by him for work on cheese, in which cylinders of the material are compressed under known loads for known times, and the strains produced are measured, being divided into elastic recoverable components and plastic permanent components.

Scott Blair(137) has extended this work, and shown, from three-dimensional curves, that freshly worked butter strain-hardens like a soft metal, i.e. for a given stress the viscosity increases with increasing strain. On the other hand, in butter which has not been worked for some time, "false body" is built up, so that the first effect of strain is to reduce viscosity, and only after prolonged deformation is strain-hardening manifest. In both cases viscosity falls rapidly, for any given strain condition, with rising stress. Although the relationship between these viscosity variations and such factors as crushing strength are not yet fully worked out, the author believes that it should be possible to interpret the spreading capacity of butter in terms of such three-dimensional diagrams, but the accuracy of the work has so far been limited by lack of adequate temperature control.

(*g*) Miscellaneous

Cooper & Hand(138) have studied the elastic and plastic properties of rennet casein from various sources. Both before and after the addition of formaldehyde, caseins from different countries of origin and casein samples prepared in different ways, differed greatly in their rheological properties. The authors compressed cylinders of casein under standard load at constant temperature for 3 min. and measured the strains, elastic as well as non-recoverable. Unfortunately, the data are not expressed in c.g.s. units in terms of viscosity and compression modulus, though the experimental procedure lends itself very readily to such an interpretation. The instrument used was described as a Scott Plastometer, but it must not be confused with the rather similar instrument made by J. R. Scott for work on rubber. For the latter instrument spheres and not cylindrical test-pieces are used.

Mellander(139) has studied the electrophoresis of casein, which material he found to consist of three clearly separable components. The P:N ratios of these separate fractions differ considerably. When casein was replaced by cow's milk dialysed against NaCl solution, a fourth phase was noted, presumed to be due to the presence of lactalbumin.

Athavale & Jatkar(140) have measured the refractive indices of oils and fats, and used this method to distinguish ghee from vegetable oils.

IV. MILK PRODUCTS

(*a*) Cream

Jackson *et al.*(141) have continued their investigation of discoloration and corrosion in canned cream, and have reported the results of further storage of their earlier batches of cream, and of storage of additional batches using different stabilizing agents. Excessive heat treatment of cream produces sulphur compounds which attack tinplate and form sulphide, the effect being enhanced by the presence of stabilizing salts added to the cream. Undermining of the sulphide film by acids causes pitting of the can and the liberated sulphide discolours the cream. The use of good tinplate, of a minimum of stabilizer—preferably sodium bicarbonate, of minimum heat treatment to ensure sterilization, and the shortest possible storage period is recommended. Nichols *et al.*(142) have shown that both bitterness and thinning in canned cream are associated with the breakdown of protein, as indicated by determinations of the peptone and non-protein nitrogen fractions.

The neutralization of cream for buttermaking has been the subject of a series of papers. Dealing with the effect of the carbon dioxide content of milk and cream, McDowall & McDowell(143) observed a general, but not regular, relationship between the acidity and the carbon dioxide content of cream. The effect of dilution with water on the titratable acidity of milk and cream next received attention, and

profiting by the results obtained by previous workers on this subject, McDowall *et al.*(144) took care to standardize the several conditions involved. The effect of dilution was shown to be due partly to the steady fall in the *p*H of the phenolphthalein end-point with increasing dilution, and partly to the dilution of the buffer substances of milk causing an increase in *p*H. The action of sodium bicarbonate on cream was studied in order to elucidate the variations in the extent of neutralization effected, under different conditions, by a given quantity of the bicarbonate, and in particular to explain the considerable variations of Valentine's factor (1 lb. sodium bicarbonate = 1·2 lb. lactic acid) from the theoretical figure (1·07 lb. lactic acid). The factor is modified by (1) the carbon dioxide content of the original milk and its reduction by any heat treatment given, (2) the higher carbon dioxide content of acid milk and cream due to bacterial action, and (3) the dilution of cream by water added as neutralizer solution. For normal creams Valentine's factor was considered valid for the neutralization of acidities of 0·12–0·20% down to 0·07–0·12%, but for high acid creams the theoretical factor was preferred(145). Townley & Gould(146) determined the efficiency with which a number of neutralizers reduced the acidity of a cream with 0·5% lactic acid to 0·25, 0·15, 0·10 and 0·05%. In general the carbonate neutralizers failed to reduce the acidity to the calculated point, the lime neutralizers were better with reductions close to the theoretical at 0·25 and 0·15%, whilst caustic soda gave the most accurate reduction.

Bird & Derby(147) examined the relationship between the loss of fat in churning and the percentages of fat and protein in the cream. They found that of the total amount of fat present in the cream the proportion appearing in the buttermilk was directly related to the initial percentage in the cream. The actual percentage of fat in the buttermilk was more closely related to the percentage of protein in the cream. Data were given for calculating the churning loss from the percentage of fat in the cream. The relationships between cream acidity, churning loss, and churning time were dealt with in a subsequent paper by Bird *et al.*(148). Creams containing 20, 30 and 37·5% fat were ripened to give a *p*H range in the buttermilk of 4·5–7·0. As the acidity increased from *p*H 7·0 to 5·5, the fat loss, calculated as a percentage of the total fat, varied little. From *p*H 5·5 to 4·7 there was a definite tendency for the fat in the buttermilk to increase when 20 and 37·5% creams were churned, but little tendency with 30% cream. From *p*H 4·7 to 4·5 the fat loss in all cases dropped sharply. With neutralized cream the fat loss was lowest between *p*H 6·5 and 7·0 rising on either side. These churning loss data correlated with the electrokinetic potentials of the fat globules.

(*b*) Butter

Composition and keeping quality

Mohr & Ritterhoff(149) have shown that sweet-cream butter has a lower protein content than acid-cream butter, the figures being 0·16–0·30% for sweet cream, and 0·4% for fine granule and 0·6–0·7% for large granule acid-cream butter. Wiley(150) has recorded graphically the figures for the titratable acidity and *p*H of seventy samples of Australian and forty of New Zealand butter of various types, salted and unsalted, and from sweet, ripened, and neutralized creams. There was a fair correlation between titratable acidity and *p*H, only five results varying by more than 0·3 *p*H from the curve drawn through the mean values. Hence either value may be used to give an indication of the acidity of the cream at the time of churning, and of the possibility of butter deterioration on storage due to excess acidity.

A high correlation between the initial flavour score and *p*H value of butter was observed by Kretchmar(151), high-scoring butters being generally less acid than low-

scoring. He gave the optimum acidity for good keeping quality of butter at *p*H 6·7–6·9, and considered it undesirable to store butter having a *p*H above 7·1 or below 6·3. McDowall & McDowell(152), when investigating the relation of the *p*H to the quality of butter, gave a *p*H of 6·7 to 7·0 for optimum keeping quality. Holm *et al.*(153) compared the rates of deterioration of butters made from creams of different titration acidities, judging deterioration by quality score, peroxide value and bleaching times. The rates of deterioration of the butters from sweet creams of up to 0·20% acidity were identical, and were much slower than for samples from creams of over 0·30% acidity. There was a direct relationship between loss of score and increase in the peroxide value of the fat. A similar relationship was found between the grade of butter after storage and the fat-aldehyde value of its fat by Wiley(154), who, having found that low *p*H and increasing salt concentration favoured the oxidation of the butterfat during cold storage, reviewed the possibility of the presence in cream and butter of a fat-oxidizing enzyme most active at a low *p*H (about 5) and high salt concentration. Concerning the effect of varying amounts of salt in butter for samples stored at 0–5° C., Bendixen *et al.*(155) observed that, when the *p*H of the butter was 6·6 or higher, the keeping quality decreased as the salt concentration in the serum increased from 9·5 to 14·2%. When the *p*H was 6·0–6·5 the keeping quality markedly improved with serum salt concentrations increasing from 7·2 to 15·4%, especially for the *p*H range 6·0–6·29.

A comprehensive study of the quality, initially and after cold storage, of butters from differently treated creams has been made by Overman *et al.*(128). Again, the sweet-cream butters showed higher initial scores and better keeping qualities than partially neutralized self-soured or starter-ripened creams. Different neutralizers, when used to adjust the acidity to the same degree, affected neither the initial score nor the keeping quality. Over-neutralization produced butter with low initial score and poor keeping quality. No connexion was found between the initial score (or keeping quality) and any of the following physical and chemical tests carried out on the fat of the butters: refractive index, iodine value, saponification value, soluble and insoluble free fatty acid content, acetyl value, Reichert, Polenske and Kirschner values, and oxygen absorption rate. The value of chemical tests for prediction of the keeping quality of butter was also investigated by Kiermeier & Maier(156). They compared the peroxide, aldehyde and methyl-ketone values obtained for butters 3 days old with the length of time the butters remained marketable when kept in cold store, and found no correlation. Mohr & Schrimpl(157) applied the following tests to the surface and deep portions of sweet- and sour-cream butters which had been stored at −20° C. for 6 months: *p*H, aldehyde, ketone and peroxide tests, refractive index and iodine value. Although sensory grading showed marked differences, these tests could not distinguish with certainty the surface and deep portions of the butter.

Many papers have appeared describing the successful use of oat-flour and its extracts in preventing oxidation of the fat when added to milk, cream and butter, and the advantage of oat-flour treated parchment as a wrapper for butter to prevent surface off-flavours(158, 159, 160, 161). Ritter & Nussbaumer(162) demonstrated a decrease in the tendency to oxidation of butterfat to which egg-, plant- and butter-phosphatides, buttermilk powder, oatmeal, or hydroquinone had been added.

During the period under review Davies(163) and Jensen & Ritter(164) have given general accounts of the factors influencing the keeping quality of butter, and Barnicoat(165) has published an informative series of papers on its packing and storage.

Aroma and flavour

In connexion with the French law of July 1935, prohibiting the addition of diacetyl as an improver to butter, Brioux & Jouis(166,167) gathered many data on the

natural diacetyl content of the butter produced in certain districts of the north of France. 76% of the samples contained from a trace to 0·5 mg./kg. of diaceytl, and the remaining samples 0·5 to 1·5 mg. with one exception (2·5 mg.). Within 15–18 days of the manufacture of the butter the diacetyl content decreased to about one-tenth of its original value. The acetyl methyl carbinol content of the butter was much higher, usually from 10 to 30 mg./kg., with a maximum of 69 mg. Pien *et al.*(168) examined many samples of French butter, and found that 42% contained less than 0·1 mg./kg. of diacetyl, 47% contained 0·1–0·5 mg., and 11% contained 0·5–1·0 mg. As no sample of butter contained more than 1·0 mg. of diacetyl, they considered that larger quantities could be attributed to artificial addition, but this conclusion is obviously invalidated by Brioux and Jouis's data given above. Parker & Shadwick(169) have discussed the production of the characteristic flavour of butter by the use of starters or butter cultures of high flavour. Considering that acetyl methyl carbinol, diacetyl, and probably butylene glycol contribute to this flavour, they suggest that the starters might be steam distilled and the volatile compounds added to butter to improve its flavour. This procedure would be preferable to the addition of diacetyl.

The formation of diacetyl during cream ripening and its development during churning were studied by Krenn(170) and Mohr & Wellm(171). After the churning process the diacetyl content of the buttermilk was 2·4–4 times that of the original cream. The butter contained one-fourth to one-sixth of the diacetyl, and one-fifteenth to one-sixteenth of the acetyl methyl carbinol content of the cream. Later Prill & Hammer(172) investigated the changes brought about in the amounts of these constituents in cream and butter by the processing and ripening of the cream. They found that butter retained from 6 to 12% of the diacetyl, and from 3 to 6% of the diacetyl plus carbinol of the ripened cream, the average values being 8·8 and 4·5% respectively.

General discussions of the chemistry of the flavour of butter and starters have been published by Mohr(173) and Davies(174).

(*c*) Cheese

During the ripening period of cheese important changes occur in the content and distribution of volatile acids. These changes, which differ markedly with the variety of cheese, have frequently been studied by liberating the volatile acids by direct steam distillation of the cheese. The question of the validity of the conclusions reached from such experiments has been raised by the important contributions of Hiscox and her co-workers to the methods of extraction of the volatile acids of cheese. Hiscox & Harrison(175) found that the direct steam distillation of cheese gave low values for the volatile acids on account of the retarding effect of the fat on the distillation of the higher volatile acids, and the permanent retention of some acids by the protein. Subsequently Hiscox *et al.*(176) tried several methods for the extraction of the volatile acids, and preferred a method based on water washing of the cheese, followed by ether extraction, and the distillation of the acids removed by these solvents. The water-soluble acids and salts that could be extracted from the untreated cheese simply by shaking with water were in excess of the total acids removed by direct steam distillation. The method finally recommended gave volatile acid values 2 and 4·5 times greater than direct steam distillation with Cheddar and Stilton cheese respectively.

There are very few published analyses of the composition of the various types of British cheese when manufactured from the same bulk of milk, and a step towards the rectification of this deficiency has been taken by Mattick(177), who has supplied comparable data on the ash, calcium, and phosphorus contents of Cheddar, Cheshire,

Leicester, Lancashire and Stilton cheese at various stages of manufacture and ripening. Remarkably small differences were found in the figures for the hard-pressed varieties, but Stilton contained much less calcium and considerably less phosphorus than any of the other varieties examined.

The role of albumin in cheesemaking has been discussed by Pyne & O'Droma (178), and Marquardt & Hucker (179) and Mattick *et al.* (180) have demonstrated that the milk from cows suffering from subclinical mastitis yields cheese of inferior quality. Krenn (181) found that curd prepared in stainless steel vats formed a spongy coagulum which adhered to the walls, unlike the curd in a copper vat which drew away from the wall and formed a desirable hard curd. The state of the steel surface due to the degree of polishing was without effect, as were changes in the acidity, temperature, rennet concentration etc. of the milk. The curd detached itself satisfactorily from the steel surface when the latter was greased with monobutyrin. Sanders & Farrar (182) showed that the presence of more than 39·7 % moisture in green Swiss cheese resulted in poor quality, especially with respect to eye formation. They listed the various factors which would increase the moisture content of the cheese.

The solubility of the protein of Cheddar cheese curd in water and in sodium chloride solutions at various times after making was measured by McDowall & Whelan (183). The solubility reached about 100 % in 3–10 % salt, within 7 days of manufacture, for both normal and high rennet cheese, and remained so. Hence, as cheese proteins were shown to be soluble in sodium chloride solutions corresponding in concentration to those found in the whole range of commercial cheeses, the effect of salt concentration on cheese quality is not due to a variation in the solubility of the protein in brine. McDowall & McDowell (184) picked out the white crystalline particles which appear frequently in mature Cheddar cheese and found, by analysis, that they were composed of calcium lactate.

The chemical changes taking place during the melting of cheese and addition of disodium phosphate or sodium citrate were studied by Kveton (185). There were no significant changes in fat constants, but the soluble nitrogen increased appreciably. The composition of the rinds from English and Dominion Cheddar cheese intended for the making of processed cheese was determined by Davies (186). The rind contained moisture 15–20 %, fat 43–54 %, crude protein 37–45 %, true protein 29–42 %, sodium chloride 1·3–2·8 %, ash 3·5–6·1 %, calcium 0·97–1·54 %, and phosphorus 0·76–0·89 %.

Annatto as a cheese colour, and the best methods for standardizing the colour, have been discussed by Carrie (187); and the chemical processes involved in cheese ripening have been reviewed by Schwarz (188).

(*d*) Other products

The moisture-absorbing powers and the changes of solubility of spray- and roller-dried skim milk and of sweet buttermilk and sour buttermilk powders, when stored at various relative humidities and temperatures, have been recorded by Davis (189), and Pien (190) has described the distinctive chemical and physical characteristics of spray- and roller-dried skim milk powder.

Kieferle (191) has outlined the preparation from skim milk of "albugen"—a by-product that is described as a valuable storable protein material which can be whipped and has many uses as a substitute for egg-white. It was prepared, under conditions not specified, by the addition of pectin to the separated milk. Two phases formed, a precipitate consisting mainly of casein, and a serum containing soluble proteins, some casein and lactose. On drying the serum, "albugen" with 98·90, 27·10 and 52·10 % of dry matter, protein and lactose respectively was obtained.

V. ANALYSIS OF MILK AND MILK PRODUCTS

(*a*) Milk

Sampling and total solids

Apparatus and methods for the sampling of milk, cream, butter, condensed milk, and cheese, and milk, whey, and buttermilk powders, have been described and specified by the British Standards Institution(192). A rapid method for the determination of dry matter in milk and dairy products has been put forward by Aschaffenburg(193), who used an inexpensive high temperature electric oven originally devised for the determination of moisture in cereals. The best conditions for total solids in milk were the drying of 1 g. samples for 6 min. at 148° C. There was no danger of loss from frothing or spurting, and there was a considerable saving of time as compared with the usual 3 hr. at the temperature of boiling water. The results agreed with the latter method, and 20 to 25 samples could be dried in less than 1 hr. Details for butter and cheese were also given.

Fat

British Standards Institution specifications of the apparatus and methods for the determination of the percentage of fat in milk, skim milk, separated milk, buttermilk, cream and cheese by the Babcock method have appeared(194); and Caldwell & Herreid(195) and Fahl *et al.*(196) have discussed the factors affecting the accuracy of the Babcock test. Filipovic(197) has criticized the accuracy of the Gerber test for fat in milk on the grounds that, using the normal 11 ml. pipette for the milk, the result is inaccurate for milk containing less than 2·5% and more than 4·0% of fat. He suggests a special pipette with three graduations corresponding with high, normal and low fat content.

Lucas(198) and Holland(199) found that the percentage of fat determined by the Babcock method in composite samples of milk was 0·1% less than the calculated average from daily tests. This was due mainly to factors associated with oiling off, churning, and packing of the fat globules, which could be prevented by the addition of 0·2% of saponin to the milk.

A new method for the determination of fat in milk, cream and ice cream has been suggested by Golding(200). After the addition of a solution containing ammonia, butyl alcohol and ethyl alcohol the fat was separated by churning to a butter, which was chilled, removed to a dish, dried and weighed.

Proteins

Improved methods for the separation of the total protein, casein, albumin, globulin and proteose-peptone substances of milk have been evolved by Rowland(201). The maximum precipitation of casein is rapidly effected from milk samples of varying casein content by the addition of 1·0 ml. of 10% acetic acid solution followed by 1·0 ml. of N. sodium acetate solution per 10 ml. of milk previously diluted. This maximum was found to be 1·0 to 1·4% greater than by the usual acetate-buffer method, and 2·4 to 3·8% greater than by the A.O.A.C. method. The methods formed the basis of a rapid semi-micro procedure for the determination of the nitrogen distribution of milk(202). The tedious transfer and washing of precipitates was avoided.

Leroy(203) has described a rapid control method for estimating the total nitrogen in milk. To 10 ml. of neutralized milk are added 1 ml. of formaldehyde sol. and 10 ml. of a standard alkali solution. The pink colour is compared with colour standards representing mg. N per litre of milk. It was claimed that in 35% of the samples

the agreement with the Kjeldahl method was within ±0·5 mg. N, and in 32% within ±1·0 mg. N. The variation was greater than 2·5 mg. N in 5% of the tests. It is surprising that a formaldehyde procedure is capable of this degree of accuracy. A representative selection of milk samples would differ widely in the proportion of the several proteins present, and one would then expect considerable variation in the ratio of total N to formaldehyde value, as found by Van der Burg & Habers(205) and Maymone & Carusi(204).

Minerals

The use of zeolites as analytical reagents for the examination of the cations of milk has been described by Davies(206). The process can be used, for example, to estimate the ionic portion of the calcium—the zeolite being charged with barium or ammonium salt—and the changes occurring therein on the addition of soluble calcium salts, acids or alkalis. The estimation of the mineral constituents of milk and its products has been discussed at length by Kagi(207). He compared the relative merits of macro- and micro-methods, and from data on the percentage errors inherent in volumetric glassware, the weighing of precipitates, titration end-points, and colorimetric comparisons, preferred macro-procedures. The advantages of wet digestion over preliminary ashing in bringing the minerals into solution were stated, and several digestion mixtures compared. Lastly he reviewed the usual methods for sodium, potassium, calcium, magnesium, phosphorus, sulphur and chlorine, gave full analytical details of his own application thereof, and indicated the degree of accuracy attained.

Miscellaneous

Wright & Anderson(208) have described a test for the presence of hypochlorite solution in milk. Chlorate is always found in the sodium hypochlorite solutions which might be used for sterilizing dairy plant, and on adding 78·4% sulphuric acid to the milk, and cooling in ice, a greenish yellow colour develops if chlorate be present. In milk 12 parts and in cream 17 parts per million of chlorine may be detected. The sensitivity of the test is increased in ultra-violet light, a strong yellow fluorescence being emitted. Bohm(209) has given detailed descriptions of colour tests for the detection of sucrose, hydrogen peroxide, formaldehyde and nitrate in milk by means of sulphuric acid. Arup(81) has investigated the pentabromoacetone method for the determination of citric acid in milk and milk products, and has pointed out that the potassium permanganate should be added gradually to avoid the formation of volatile substances which interfere with drying to constant weight.

The detection of neutralized milk by the Tillmans-Luckenbach method has been tested by Leyko & Cieplisnka(210) with good results. The number of ml. of $N/4$ sodium hydroxide required to overcome the buffering action between *p*H 3·2 and 8·4 of a colloidal iron filtrated from 100 ml. of milk was always less than 1 ml. for unneutralized milk. The available methods for the detection and determination of neutralizers in milk and dairy products have been described and discussed by Davies(211). The Evenson colour test for remade milk has been modified by Fairbanks *et al.*(212), who showed that by improved washing of the protein and the use of a stronger sodium hydroxide solution, a more striking contrast between natural and remade milk is obtained, and the detection of 5% of remade milk is rendered possible.

A method for the differentiation of cow's, goat's and sheep's milk has been suggested by Buogo & Zonno(213) based on the "iodine number" of the milk, which was defined as the number of g. of iodine absorbed by 100 ml. of milk from Hubl's iodine solution in 4 hr. Mantovani(214) detected the addition of cow's milk to goat's milk by incubating with the Schardinger reagent. Unheated cow's milk decolorizes it rapidly; goat's milk does not.

(*b*) Butter and butterfat

The British Standards Institution (215) has specified methods for the determination of moisture, fat, curd and salt in butter, and of the Reichert, Polenske and Kirschner values for the volatile acids in the fat. The following supplementary special tests are also given: curd-protein, lactose, acidity, sodium chloride, copper and iron in the butter; the iodine value, saponification value, refractive index and peroxide oxygen of the fat; and the hydrogen ion concentration in the curd serum.

Dichlorofluorescein, as an adsorption indicator, has been shown to be preferable to the usual potassium chromate indicator in the direct titration of sodium chloride solutions from butter with silver nitrate. It gives a sudden change from a greenish yellow fluorescence to a distinct pink colour, and renders the estimation easier and more accurate (216, 217).

Chromatographic adsorption analysis provides a reliable method for the detection of artificial colouring of butter (218). Pure butter adsorbed with alumina gives a faint but distinct chromatogram, artificial fat and butter colours give a different chromatogram and small additions of these can easily be detected.

Ritterhoff (219) has described a rapid colorimetric method for the approximate estimation of the iron and copper content of butter, and Smith (220) a rapid method for estimating the peroxide values of the fat in whole milk powders, the values being only about 90% of the true values, but useful for comparative purposes. Breazale & Bird (221) have described and compared the available methods for the titration of the free acids in butterfat, and the separation of these acids from the fat.

Erlandsen (222) has made a thorough detailed review of recent literature on the composition of butter and margarine, and given special attention to methods for determining the proportions of each in a mixture of the two.

(*c*) Cheese

Methods for the routine determination of moisture, fat, and salt in cheese are described by the British Standards Institution (223), and tests for *p*H and titratable acidity included. The specification recommends the Schmid-Bondzynski-Ratzlaff method for fat, which method, incidentally, has been criticized by Florentin (224) on the grounds that the hydrochloric acid used to liberate the fat causes partial decomposition of the fat and low results. However, Swaving (225) later demonstrated that the criticism was unjustified. Gould (226) has continued the comparison of various methods for determining the moisture content of Cheddar cheese.

To separate the extraneous matter in cheese, Spicer & Price (227) make an emulsion of the minced cheese by heating with hot sodium citrate solution. This is filtered through a cotton wool pad, which is washed with water to reveal the sediment. The detection of benzoic acid and several of its derivatives when present as preservative in process cheese, is the subject of a paper by Schwarz & Kahlert (228).

Garard *et al.* (229) determined the Polenske value of the extracted fat to distinguish blue cow's milk cheese from the more expensive ewe milk Roquefort cheese. Whereas the Polenske value for the cow's milk cheese was not more than 2·9 and usually 1·8, for the ewe's milk cheese it ranged from 3·60–5·95. The extracted fat from the ewe's milk cheese was always pale green in colour, and from the cow's milk cheese deep yellow.

(*d*) Miscellaneous

The advantages of the toluene distillation over the oven-drying method for the determination of moisture in dried milk products have been enumerated by Sharp *et al.* (230), who pointed out that dried skim milk and whey containing lactose in the

crystalline α-hydrate form require longer for the removal of water than similar products with lactose in the glassy β-anhydride form. A test for measuring the extent to which cream will oil-off in coffee has been devised by Jenkins & Mack(231); water at 200° F. being added to 1 ml. of cream in a Babcock skim milk bottle which is centrifuged and the amount of fat taken as a measure of the power to oil-off. Epple & Horrall(232) have described a method for the extraction, for purity tests, of the fat in ice cream. Acetic acid and ethyl alcohol were used, as preferable to the usual ammonia or sulphuric acid. Munsey(233) has reported a comparison of the results of the citric acid, fat number and lactose methods for the estimation of milk solids in milk bread. The citric acid method gave results which were reasonably near to the true values.

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